The role of epicuticular components in the pathogenesis of metarhizium anisopliae var. acridum

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Award date:
2001

Awarding institution:
University of Bath

The role of epicuticular components of the desert locust, *Schistocerca gregaria*, in the pathogenesis of the entomopathogenic fungus *Metarhizium anisopliae* var. *acridum*

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2001

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Dedicated to

Ernest Stringer,

my grandfather

and a scientist

The role of epicuticular components of the desert locust, *Schistocerca gregaria*, in the pathogenesis of the entomopathogenic fungus *Metarhizium anisopliae* var. *acridum*

Submitted by S. L. Jarrold

for the degree of Ph.D.,

University of Bath,

2001.

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iii Abstract

Germination and appresssoria formation of the entomopathogenic fungus *Metarhizium anisopliae* var. *acridum* was studied *in vitro* on ¼ strength Sabouraud's dextrose agar (¼ SDA) and semi-*in vivo* on excised wings of the desert locust, *Schistocerca gregaria*. Conidia started to germinate at 12h post-inoculation on ¼ SDA and 8h post-inoculation on sterilised locust hind wings. Appressoria first formed 18h post-inoculation on both ¼ SDA and standard-preparation hind wings. However, appressoria appeared significantly more slowly on hind wings from locusts reared under axenic conditions, suggesting that the microflora may alter the surface of the cuticle in such a way as to promote germination.

Tween 80 and Ondina El oil supported significant germination in the fungus, so were not used as formulation additives during experiments.

Significantly less germination and appressoria formation occurred on wings of non-host *Calliphora vomitoria* than on host insects (locusts). Thus, pre-penetration events may contribute to host specificity. On non-host *Tenebrio molitor* wings, germination was lower, but appressoria formation was higher, suggesting the presence of a trigger to appressoria formation on the surface *T. molitor* wings

The chemical composition of the hind wing epicuticle was determined by pentane, chloroform and methanol extraction. The greatest levels of germination were observed on the methanol extract, with negligible germination on pentane and chloroform extracts. The methanol extract was shown by GC-MS analysis to consist primarily of free fatty acids and esters. TLC was used to further separate the methanol mixture. One band that contained linoleic acid supported the highest level of germination. Germination was good on an authentic free fatty acid (oleic acid), but significantly less than on linoleic acid taken from the wing. Long chain authentic

n-alkanes presented alone to the fungus supported excellent germination in the fungus, despite having little impact when presented as a mixture in the pentane wing extract.

The methanol extract contained monosaccharides and proteins as well as lipids. The fungus germinated in similar concentrations of authentic glucose and amino acids to those found in the locust wing extracts.

A technique was developed that enabled complete removal of fungus from the wing after appressoria formation but before penetration. This involved hydrolysis of fungal mucilage with laminarinase. The composition of the hind wing epicuticle extracts was determined before and after treatment with fungus. Some compounds were completely absent from fungus inoculated wings, indicating that they had been used by the pathogen, whilst others were reduced. 83.7% of available octacosane was utilised during 24 h exposure to fungus.

The chemical analysis of the hind wing epicuticle pre- and post-fungal treatment was complemented by structural studies using Cryo-Scanning Electron Microscopy (Cryo-SEM) and Atomic Force Microscopy (AFM). Cryo-SEM showed epicuticular 'clearing zones' on areas where fungus had been removed. AFM revealed for the first time the effect of a fungus on the surface architecture of the epicuticle of an insect.

iv Abbreviations

l - litre

ml - millilitre

µl - microlitre

cm - centimetre

mm - millimetre

nm - nanometre

g - gram

mg - milligram

µg - microgram

SDA - Sabouraud's Dextrose Agar Medium

mag. - magnification (light and electron microscope)

TLC - Thin Layer Chromatography

GC-MS - Gas chromatography - mass spectrometry

SEM - Scanning Electron Microscopy

AFM - Atomic Force Microscopy (= Scanning Probe Microscope)

IMI - International Mycological Institute

RH - Relative Humidity

LUBILOSA - Lutte Biologique contre les Locustes et le Sauteriaux

FAO - Food and Agriculture Organisation of the United Nations

ULV - Ultra Low Volume

***N*-A-G** – *N*-acetyl glucosamine

v Acknowledgements

I would like to thank the following:

- Very special thanks to Andrew Campbell for his support and faith in me,
- My family Carol, Dennis and Ian Jarrold
- Dr. A. Keith Charnley (Supervisor, University of Bath)
- Dr. John Clarkson and Prof. Stuart Reynolds (Assessors, University of Bath)
- CABI Bioscience- Dr. Dave Moore (Supervisor, CABI Bioscience), Dr. David Dent, Dr. Roy Bateman, Belinda Luke
- Dr Chris Jackson and Prof. Paul Jepson– who sparked my interest in Entomology and biocontrol
- Dr John Fox, Alta Bioscience – University of Birmingham, amino acid analysis
- Royal Entomological Society – Prof. Roger Blackman, Dr. Richard Harrington, Prof. Richard Wall, Dr. Les Allen-Williams, Greg Bentley and Bill Blakemore – members of Council of which I was a Trustee and Member, 1997-2000
- Department Pharmacy – Dr. Terry Jefferies, Kevin Smith, Ryan; GC-MS
- Department Chemistry – Prof. Malcolm Sainsbury, development of extraction regime
- Department Material Sciences – Mrs. Ursula Potter, Cryo-SEM, AFM
- James Kerwin, Keith Jones, Chris Lomer, Mark Goettel, Don Roberts, Randy Gaugler - VIIIth International Colloquium of the Society of Invertebrate Pathology, Sapporo, Japan
- My friends Hannah Jenkins, Cheryl Mansbridge, Rhian Essex
- Department Biology and Biochemistry and MPG – Dr Sarah Backen, Catherine Cheeseman, Dr. Graeme Down, Janet Graystone, Dr. Natasha Bye, Dr. Rod Dillon, Xia.
- This work was supported by the BBSRC and CABI Bioscience

Thank you for all your help and support!

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1 Introduction

'The role of insect epicuticular components in the pathogenesis of the deuteromycete, *Metarhizium anisopliae* var. *acridum* for the desert locust, *Schistocerca gregaria* (Forskål)'

Fungal entomopathogens have been of interest as agents of biological control of insect pests for more than one hundred years (Moore and Prior, 1993), but due to problems encountered in areas such as specificity, mass production, speed of kill, formulation and storage of the live pathogen, potential for practical insect control has yet to be fully realised.

One key exception has been the development of *Metarhizium anisopliae* var. *acridum*, a Deuteromycete fungal pathogen for control of the desert locust, *Schistocerca gregaria*. *M.anisopliae* var. *acridum* has recently been launched as a commercial product under the name 'Green Muscle®' (Bateman *et al.*, Green Muscle® User Handbook, 1998). This mycoinsecticide meets the highest standards and specifications (Bateman *et al.*, Green Muscle® User Handbook, 1998) and is the result of a multi-national, multi-donor research and implementation programme, LUBILOSA (Lutte Biologique contre les Locustes et le Sauteriaux), which began in 1989. One of the critical players in this programme, CABI Bioscience, was the Industrial sponsor of this research.

The object of this project was to investigate the early stages of the insect/entomopathogen relationship, in particular the contribution of epicuticular chemicals, to germination and appressorial formation by the fungus. Studies of fungal pathogenesis may provide information that will lead to increased speed of kill through formulation additives and improved storage. An understanding of the basis of specificity in the pathogen may allow a rational approach to improvement. The ultimate aim would be the application of any novel discovery to the production of a more efficient, commercial biological control product, which could provide a match for the performance and efficiency of chemical pesticides. Despite being environmentally unfriendly, chemical pesticides remain the mainstays of desert locust control today.

LITERATURE REVIEW

1.1.0 Fungal entomopathogens as agents of biological control

Entomopathogenic fungi can be found throughout the fungal kingdom, but most are Zygomycotina or Deuteromycotina (Ferron, 1978). Virtually every major fungal taxonomic group except the higher Basidiomycotina has entomopathogenic members. Approximately 90 fungal genera and 700 species are entomopathogenic (Roberts and Humber, 1981) of which only 10 have been, or are at present being developed as agents of biological control (Hajek and St. Leger, 1994).

Entomopathogenic fungi vary in host specificity, some species being more restricted (*e.g. Aschersonia aleyrodis* only attacks scale insects) whilst others infect a wider

host range, with specificity at the variety or isolate level, for example, *Metarhizium anisopliae.* Some isolates of for example, *M.anisopliae* var. *anisopliae* are pathogenic for soil pests and have been developed to control the pasture cockchafer, *Aphodius tasmaniae. M.anisopliae* var. *majus* is used in south-east Asia and the Pacific against the rhinoceros beetle, *Oryctes rhinoceros* (a coconut pest) (Gillespie and Claydon, 1989), and *M.anisopliae* var. *acridum*, isolate IMI 330189, (the object of the present study) is exclusive to certain locust and grasshopper hosts (Bridge *et.al.*,1993).

Fungi have been put forward as agents for biological control of insects for over a century. Attempts have been made to use fungi as mycoinsecticides since 1836, when Agosto di Bassi discovered that a white muscardine disease found in silkworms (*Bombyx mori*) occurred as a result of a fungal infection, later named *Beauveria bassiana* (Gillespie and Claydon, 1989), but success has been limited. Efforts have mainly focussed on entomopathogenic species such as *Verticillium lecanii, B. bassiana, A. aleyrodis, M. anisopliae* and *Nomuraea rileyi* (Clarkson and Charnley, 1996) as agents of biological control. At present fungi like these are being used in China against scale insects, in Brazil against sugar cane spittle bugs, and commercially in greenhouse environments in certain parts of Europe to control whitefly and aphids (*e.g.* Mycotal® and Vertalec®, based on *V.lecanii* spores produced by Koppert B.V.).

A key reason often cited for the variable efficiency of fungal pathogens is their requirement for high environmental humidity. For example, in Deuteromycete fungi such as *M. anisopliae*, the minimum relative humidity (RH) for conidial germination

and further mycelial growth is 93%, but the optimal RH for rapid germination and growth is 98-100% (Gillespie and Claydon, 1989). This problem can be overcome, however, by the use of oil formulations, as shown for *M.anisopliae* var. *acridum* against the desert locust, *S.gregaria*, allowing infection at very low humidities (Bateman *et al.*, 1993).

Although fungal pathogens do not kill insects as rapidly as chemical insecticides, they are specific, environmentally friendly and hold the potential for self-perpetuation. This means the disease can spread through the contact between other pest individuals with a sporulating cadaver in the natural environment of the pest (see section1.3.2.). It is also worth noting that fungi have the same potential to adapt to their hosts, as their hosts have to develop resistance (a growing concern involving chemical insecticides).

1.2.0 The host

1.2 1. The desert locust, *Schistocerca gregaria* (Forskål)

The desert locust is an orthopteran belonging to the family Acrididae. It is one of 12 species of short-horned grasshoppers (Acridoidea) that exhibit the prime locust characteristics of being large in size and possessing the ability to change behaviour when large numbers occur.

The desert locust exists in two forms, solitary and gregarious (Uvarov, 1966), and up until 1921 it was thought that *S.gregaria* was actually 2 different species (FAO Desert Locust Information Service, http://www.FAO.org)! Solitary insects are

relatively inactive, exhibit homochromy viz. they adopt the colour of their environment. In practice this means juveniles vary in colour between dark green and brown. The adults come together only when mating and fly mainly at night. Gregarious juveniles are yellow with black markings, and develop into pink (immature) and yellow (mature) adults which gather into day flying forms of billions of insects. The change from solitary to gregarious is thought to occur in the wild after desert rains produce a flush of vegetation. Researchers have suggested that a chemical signal produced in the foam surrounding locust eggs when a female has been in crowded conditions resulting in physical contact with other locusts, is responsible for the phase change (Tickell, 1996). When the foam was removed from locust eggs that would normally have hatched into gregarious young, the locusts were born solitary. If the same foam was applied to eggs that would otherwise have hatched into solitary locusts, they were born gregarious.

In the gregarious phase gregarious adults form swarms, and marching, wingless juveniles form hopper bands. Prolonged periods of locust swarming simultaneously over large areas of Africa result in plagues. Swarms and bands can be dense and highly mobile. Winged adults can form swarms from <1 to several hundred square kilometres in size, and each square kilometre can contain 20-50 million insects weighing about 40 to 100 tons (Courshee, 1990). Each locust in a swarm is capable of consuming its own weight in vegetation each day. One square kilometre of a locust swarm can consume an equivalent amount of food to 2,500 people per day (NRI Locust Handbook, 1990). This, together with the wide variety of food consumed, results in incredible devastation in areas covered by a swarm.

1.2.2. Where and how do swarms form?

Desert locust plagues may last for many years and are characterised by many swarms of adults and large numbers of hopper bands distributed throughout the infestation area. Migration of swarms also occurs between seasonal breeding areas. Plagues are separated by recessions where infestations of gregarious locusts are few, small and transitory. A plague usually begins as an upsurge, and as an upsurge develops the bands become progressively larger and more cohesive. This leads to gregarious behaviour and breeding becomes more successful with successive generations (Symmons, 1992). Plagues usually end naturally as a result of catastrophes. The last significant plague ended in 1988 with a spectacular migration of locusts from Africa to the Caribbean after a failure of rains.

During recession periods desert locusts are restricted to semi-arid deserts of Africa, the Near East and south-west Asia, areas which receive <200 mm rain annually. This area covers ~16 million km^2 and consists of ~30 countries (Cheke and Holt, 1993). At times of plague desert locusts can spread over the enormous area of 29 million km^2, and swarms can occur in parts of 60 countries. This area is equivalent to 20% of the total land surface of the world, and the devastation can affect the livelihood of a tenth of the world's population (FAO Desert Locust Information Service, http://www.FAO.org). Plagues of locusts have been reported since the Pharaonic times in ancient Egypt (http://www.FAO.org, 1999). During the last century, *S.gregaria* plagues occurred in 1926-1934, 1940-1963, 1967-1969 and 1986-1989.

The last plague to affect countries from West Africa to India occurred in Western Sudan rapidly increased in number in Western Sahara in late 1987 and then went on the infest the Sahel, Arabia and South West Asia (Skaf *et al.*, 1990). The plague ended in 1989 as a result of massive control operations in which ~15 x 10^6 l of ultra low volume (ULV) formulation pesticides (for example fenitrithion, malathion and deltamethrin) were used (Symmons, 1992) and due to the mass migration described above.

The most recent upsurge affected countries along the Red Sea in 1996-1998. It developed as a result of a cyclone in June 1996 and heavy rain in November of that year. Infestations of the desert locust were concentrated in Saudi Arabia, Egypt, Eritrea, northern Somalia, Sudan and Yemen. The upsurge was brought to an end as a result of large-scale control operations in Saudi Arabia in 1998.

A locust alert was issued by the Food and Agriculture Organisation of the United Nations (FAO) in August 1999. Swarms of locusts began to spread from their traditional breeding grounds of Kazakhstan to the Russian federation, Uzbekistan and Kyrgystan, areas of which reported locust infestation for the first time in 70 years. 1.1million hectares of crops such as grains, pulses, sunflowers and soybeans were reported as damaged. Reports of desert locust eggs being laid over 9 million hectares of land gave rise to fears of an even larger infestation in 2000. The locust infestation added to an ongoing crisis in agriculture in these countries, caused by lack of capital, currency devaluation, drought and early frost, and more extreme infestations in 2000 could cause serious problems for food production in the affected region (FAO Special News Alert, 1999).

1.2.3. Strategies to combat the desert locust

Desert locust plagues have been difficult to control in the past, due to many factors, summarised below:

- The extremely large area covered by a plague (16-30 million km^2)
- The remoteness and difficult access to plague areas (often inhospitable terrain)
- The lack of safety in some areas (e.g. land mines)
- The limited resources for locust monitoring in affected areas
- The undeveloped basic infrastructure (roads, communications, water, food) of some countries affected by plagues
- The difficulty of maintaining a sufficient number of trained staff and resources during the long periods of recession between unpredictable plagues
- Political relations among affected countries
- The difficulty of organising and implementing control operations where pesticide must be applied directly to locust swarms
- The difficulty in predicting outbreaks (lack of periodicity of plagues, uncertainty of rainfall)
- Lack of funding for expensive control programmes (plagues usually occur in underdeveloped countries

The mainstay of desert locust control has been the use of chemical pesticides. Persistent insecticides such as dieldrin were widely used to eradicate hopper bands. With a fully cumulative toxicity of 5 $\mu g\ g^{-1}$ (Courshee, 1990) dieldrin, inoculated onto the vegetation in concentrations of a few micrograms per gram of vegetation, would kill a locust after a couple of days of feeding. Barrier spraying targeted later

instar hopper bands and took advantage of the consistency in distance moved demonstrated by these bands (~ 1 km each day). Dieldrin was placed on 100m wide strips of vegetation across the predicted path of the hoppers, whilst 2km areas between the dieldrin strips were left free of pesticide. Both methods were thought to be very successful and upsurges were prevented from developing into plagues over the 30 years of dieldrin use. Both techniques were designed to increase the work rate of an aeroplane sprayer. However, these persistent chemicals were banned ca. 15 years ago due to their cumulative toxicity to non-target organisms.

The present method of controlling desert locust swarms and hopper bands is the application of mainly organophosphate chemicals, such as fenitrothion, malathion, chlorpyrifos, carbaryl, phoxim, bendiocarb and propoxur. Although these non-persistent chemicals are not as poisonous as persistent pesticides, they are less efficient and more expensive. They are applied as small, concentrated doses in an Ultra Low Volume (ULV) formulation, by vehicle-mounted and aerial sprayers and sometimes by knapsack or hand-held sprayers. Non-persistent organophosphates can also be targeted at swarms as well as hopper bands, although hopper control has remained the chief technique for combating a plague. During the last plague of desert locusts (1986-1989) US$200 x 10^6 was provided by donor countries towards the cost of these pesticides, whilst the affected countries contributed an equal amount. This huge effort had doubtful impact (Symmons, 1992), and may, like the persistent insecticides, have had a harmful effect on the environment. Such considerations prompted a search for alternatives to chemical insecticides.

1.2.4. Future desert locust control

Improved methods of locust control are sorely needed. The FAO of the UN currently spends US$350 million a year on locust control, primarily on insecticides (Tickell, 1996).

Many alternatives to insecticides have been researched. The current focus is mainly on biological control using natural enemies and insect growth regulators. These agents have been considered for stand-alone control and as part of integrated pest management schemes combined with small doses of chemical insecticides. The criteria for a new form of locust control included 1) simple and inexpensive production methods, 2) safety to non-target organisms (host specificity), 3) fast acting, and 4) safety for the environment.

Locusts are killed by a wide range of natural enemies including predatory insects, protozoa, mermithid nematodes, many mammals and birds, one recorded baculovirus and Deuteromycete fungi such as *Metarhizium* and *Beauveria* species (Prior and Lomer, 1991). These enemies reduce the multiplication rates of locusts in their *natural* environment, but they do not prevent locust plagues. Although pathogens of the desert locust had received little attention in the past, their potential as biological control agents was recognised in 1989, when a collaborative research programme (LUBILOSA project) was launched to investigate the feasibility of control of desert locusts using pathogenic fungi.

Species of fungi in the genera *Beauveria* and *Metarhizium* are pathogenic to locusts, whilst being non-pathogenic to vertebrates. Both have the advantage of host-specific

isolates, and are produced on simple substrates such as cereal grains. Spores from these pathogens have also been formulated for application with conventional ULV spray apparatus. Fungal pathogens gained advantage over other natural enemies such as viral or bacterial pathogens, due to their being contact pesticides, penetrating the insect cuticle directly. The pathogenic spores do not have to be ingested so problems associated with product life and survival of baits and sprays in the harsh desert locust environment are minimised.

Other methods which have been put forward as alternatives to chemical control of locusts include the use of feeding deterrents (Gill and Lewis, 1971) where an unpalatable substance was placed onto the surface of vegetation to discourage feeding in the *S.gregaria.* Researchers are considering the use of the chemical component in the foam secreted onto desert locust eggs (causing the hatchlings to be gregarious) to encourage swarming in the absence of sufficient vegetation, so starving the locusts (Tickell, 1996). If the chemical component of the foam around desert locust eggs could be identified, the area of production could be targeted to stop production and prevent the change of phase from solitary to gregarious (gregarious behaviour leads to swarming). Other researchers suggest that the non-persistent chemicals should remain in use, but that strategies of their use be improved through a better understanding of plague dynamics. Symmons (1992) suggests three possible strategies for better control:

1) to prevent plagues by control during the upsurge stage
2) to eliminate plagues by destroying nearly all of the locusts, attacking swarms rather than hopper bands as insects in flight receive a greater coverage of insecticide

3) to concentrate on protecting the crops and allow the plague to follow its natural course.

1.3.0. The insect integument

1.3.1. Functions

The integument of insects (and the phylum arthropoda as a whole) consists of the basal lamina, cellular epidermis and extracellular cuticle (Gillott, 1995), and serves as far more than an exoskeleton (Hepburn, 1985). The integument undergoes great structural, chemical and physical changes during the life cycle of an insect, and its types and qualities of layers come and go with every instar. Such changes give rise to many important general properties and functions, summarised below. The integument provides:

- protection of internal organs (Hepburn, 1985)
- a rigid structure for attachment of muscles (apodemes) (Neville, 1975, Reynolds, 1989, Gillott, 1995)
- jointed limbs (Neville, 1975)
- a barrier against water loss (Beament, 1961) (very significant to the success of this class in terrestrial evolution, Gillott, 1995), and against predators, parasites and pathogens (Locke, 1984, Reynolds, 1989)
- appendages, such as hairs, bristles, wings, mouthparts and antennae (Neville, 1975)
- flight ability in some insects (Gullan and Cranston, 2000)
- jumping in some insects (Neville, 1975)
- moulting (Weis-Fogh, 1970, Reynolds, 1989)

- lining and control of the tracheal system (Neville, 1975)
- sound production in some insects *e.g.* stridulatory file of locusts and grasshoppers (Neville, 1975)
- construction of sense organs (Reynolds, 1989), scales and colours (Neville, 1975)
- structural releasers of behavioural sequences in insects, *e.g.* some cuticular hydrocarbons which serve as chemical sex pheromone molecules (Gillott, 1995)

1.3.2. Structure of the insect integument

At its simplest, the integument is a multi-laminar structure, containing many different bound lipids, hydrated polymers, free waxes and proteins. The integument, however, is not a uniform structure and its cellular and extracellular components are differentiated according to the function it is required to perform (Hepburn, 1985, Gillott, 1995). The structure of the integument, the complexity of which has been the subject of much controversy [Wigglesworth (1976), Weis-Fogh (1970), Locke (1984), Hepburn (1985)], is reviewed, from inside to outside the insect, below.

1.3.2.1 BASAL LAMINA

The basal lamina contains carbohydrate and collagen-like material and is secreted mainly by the epidermis, although haemocytes have also been linked with its production. This layer can be up to 0.5μm and is amorphous but selectively porous (Gillott, 1995). At metamorphosis processes from the overlying epidermis (see below) extend into the basal lamina. When the processes shorten, the basal lamina buckles and so brings about the rearrangement of cells which allows a larva to become a pupa (Gillott, 1995).

1.3.2.2 EPIDERMIS

The epidermis is a virtually continuous single-celled layer between the basal lamina and the cuticle (Gullan and Cranston, 2000). It is responsible for secreting the majority of the overlying cuticle (Reynolds, 1989, Gillott, 1995), including structural components, waxes, cements and pheromones. When actively secreting, epidermal cells change shape from flat to cuboidal, and extensive rough endoplasmic reticulum and Golgi apparatus (fine structure synonymous with secretory activity in cells) are readily visible under the electron microscope. Numerous pore canals traverse the cuticle secreted by the epidermis. The canals branch into numerous finer wax canals, which terminate at the surface and deliver lipids from the epidermis to the surface of the insect (Gullan and Cranston, 2000).

Epidermal cells can also form connections with other structures via cytoskeletal extensions. Gillott (1995) cites the use of cytoskeletal extensions to draw tracheoles closer to cells in order to increase oxygen supply.

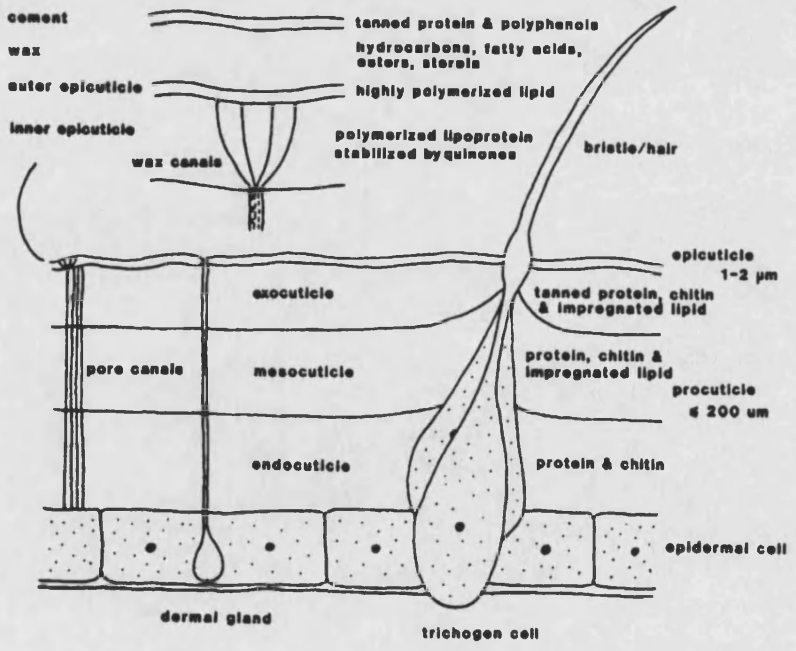
Some sense organs and glandular cells, such as dermal glands and oenocytes, are produced through differentiation of epidermal cells. Dermal glands are unicellular and have a long duct leading to the exterior of the insect through the cuticle. Dermal glands produce cement and/or wax, whilst pairs or clusters of oenocytes, situated on the haemocoelic face of the basal lamina or within the fat body after migation from the integument. Oenocytes have been linked to the production of cuticular hydrocarbons (Diehl, 1975, Blomquist and Dillwith, 1985) and in the production of ecdysone.

1.3.2.3 CUTICLE

The cuticle is thin but very complex (Gullan and Cranston, 2000) and can be differentiated to suit an insect's functional needs. For example, the cuticle is thick at points of muscle attachment, yet elastic and flexible where movement occurs (Gillott, 1995).

The cuticle covers the entire body surface of an insect including the tracheoles, foregut and hindgut, with the exception of the chemoreceptors (to allow entry of stimulant molecules), midgut (to allow for digestive absorption) and end gland cells (for excretion). Its general structure consists of a thicker procuticle overlaid with thin epicuticle, (Fig. 1.1, Hepburn, 1985).

Figure 1.1: General structure of insect cuticle (reproduced from Hepburn, 1985)



1.3.2.3.1 EPICUTICLE

The epicuticle is inextensible and unsupportive, and is responsible for delimiting the size of an insect (Gullan and Cranston, 2000). It can be between 0.1 and 3μm thick, and is made up of as many as five layers; an inner epicuticle, an outer epicuticle, a superficial layer, a wax layer, and a cement layer (Hepburn, 1985, Gullan and Cranston, 2000). It has also been inferred that the epicuticle acts as a chemical reservoir for storage of waste metabolic products (Neville, 1975).

1) The Cement Layer.

The cement layer is not always present in every insect epicuticle, or on every part of an individual. Distribution and thickness of the cement layer can vary enormously. Literature concerning this layer of the epicuticle is scarce (Hepburn, 1985) and therefore calls for more research. It is known, however, that the cement is produced by combined secretory products of specialised dermal glands and it has been suggested that it consists mainly of wax stabilised with shellac, with a degree of tanned proteins present (Neville, 1975). The cement layer, when present (*e.g.* in locust cuticle), overlies the wax layer and provides protection against abrasive damage.

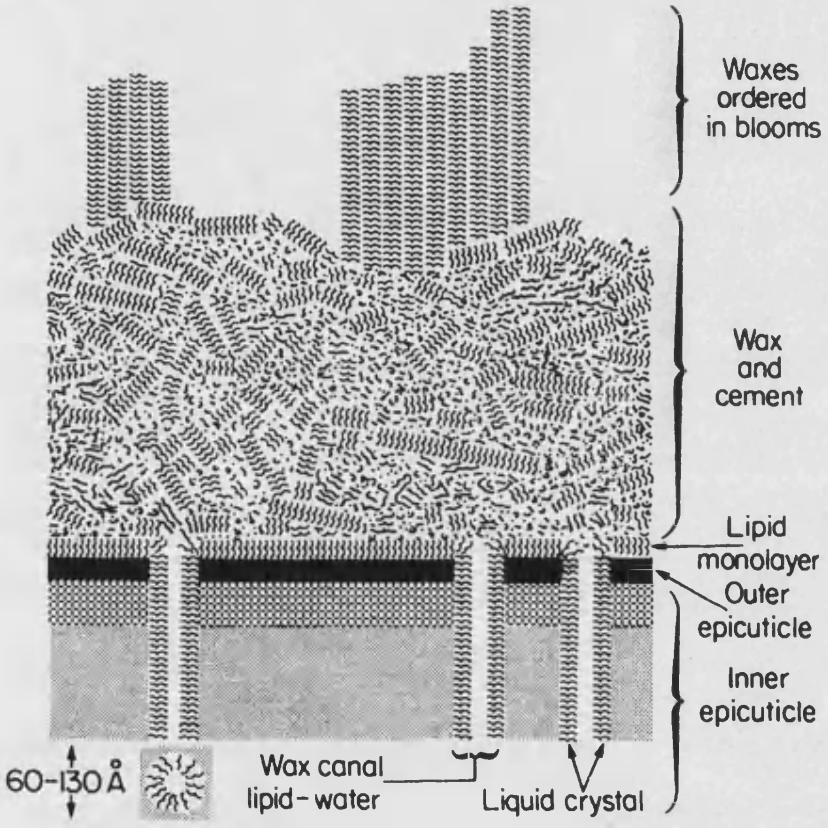
2) The Wax Layer

The wax (lipid) monolayer lies beneath the cement layer or forms the outermost layer in insects lacking the cement layer. The wax layer is secreted via wax canals, which fuse to become pore canals as they reach the cuticle surface (Gullan and Cranston, 2000). In areas where pore canals are present, mobile liquid crystalline wax flows or is groomed by the insect to cover the surface (Gu *et al.* 1995). Other functions of the

wax layer, besides controlling the water permeability of the cuticle, include the protection of the cuticle (through the hardness of the wax), the presence of pheromone molecules (volatile hydrocarbons present in the wax layer, Blomquist and Dillwith, 1985, Udayagiri *et al.*, 1997), and the possible antimicrobial properties of wax layer components (Schabel, 1976). Hydrocarbon profiles of the wax layer could also hold very important information for taxonomy of insects (Grunshaw *et al.*, 1990, Anyanwu *et al.*, 1997).

The arrangement of the waxes within the wax layer is not certain to date, but huge variation is thought to exist (Hepburn, 1985). Locke (1984) reported that the wax and cement layer may be combined in some insects (*e.g.* Heteroptera and Homoptera), where wax blooms extend above the surface cement layer (see Figure 1.2).

Figure1.2: The arrangement of epicuticular waxes as suggested by Hepburn, (reproduced from Hepburn, 1985)



The composition of the wax layer has been found to differ with species, individuals, age, sex, under dry conditions (Gullan and Cranston, 2000) and according to whether the insect is diapausing or not. Hydrocarbons are generally the most dominant component, whilst alcohols and esterified fatty acids are also present (Oraha and Lockey, 1990). The components of the insect epicuticle are more thoroughly reviewed in section 1.4.0 concerning insect lipids.

3) The Outer Epicuticle

The outer epicuticle is a trilaminar membrane measuring 180Å in thickness. Its occurrence is universal in epicuticle and it is the first formed layer of the integument at moulting (see section 1.6.3). The chemistry of the outer epicuticle is unknown (Locke, 1984, Hepburn, 1985), apart from that its resistance to degradation suggests properties similar to polythene-like polymers, and to quinone-tanned proteins. It is also perforated by pores (Locke, 1984) which may provide a selectively permeable barrier for cuticle to be reabsorbed, and is the site of muscle attachment fibre insertion. The outer epicuticle is also important in surface pattern formation, as buckling of this layer gives rise to structures on the cuticle surface (Neville, 1975) and microsculpting of this layer can create various other cuticular patterns, such as light reflectivity (Gullan and Cranston, 2000). Resistance to moulting fluid enzymes is an important property at the moult, because it prevents hydrolysis of the new cuticle along with the old.

4) The Inner Epicuticle

Wigglesworth (1937, see 1976) proposed that this layer is a reservoir for the extracellular enzyme polyphenol oxidase, which is associated with wound repair in

the cuticle (see section 1.3.4 below). Polyphenol oxidase is responsible for tanning abrasions or scratches that occur in the cuticle. The inner epicuticle can be 0.5-2μm thick, and Weis Fogh (1970) suggested it comprises of polyphenols. This layer is a secretory product of the epidermal cells together with contributions from oenocyte cells (Wigglesworth, 1976).

1.3.2.3.2 PROCUTICLE

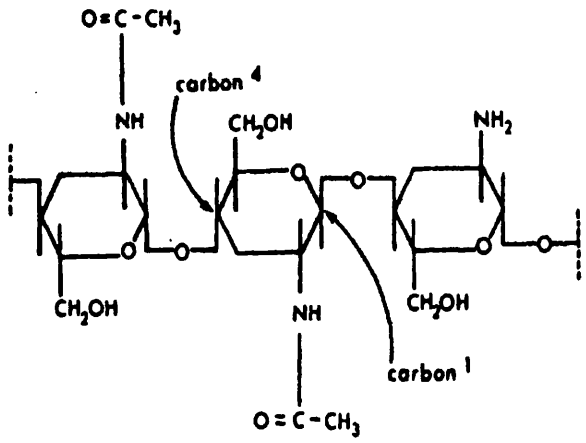
The procuticle (fibrous cuticle) provides strength and support to the insect integument. It forms the bulk of the cuticle and has two main divisions known as the thick endocuticle and thinner exocuticle (Gillott, 1995, Gullan and Cranston, 2000). The exocuticle differs from the endocuticle in that it is sclerotised to add extra strength and hardness. Sclerotization is an irreversible process that occurs when adjacent protein chains in the exocuticle become linked by phenolic bridges (known as 'quinone tanning') or by dehydration of the protein chains (Gullan and Cranston, 2000). Exocuticle is not present in areas of the integument where flexibility is needed, e.g. intersegmental membranes and joints (Gillott, 1995). In some species and intermediate area called mesocuticle, exists between the endocuticle and exocuticle (see Fig. 1.1). Different types of procuticle are found performing different functions at various sites on the insect cuticle. The most common procuticle type is **solid** cuticle, which occurs in mouthparts and the sclerites of hard-bodied insects. It is strengthened through sclerotisation – an irreversible process where the exocuticle is quinone-tanned so that the proteins become insoluble (Gullan and Cranston, 2000). A different type of procuticle is found between sclerites, called **arthrodial** membrane. This type of cuticle is tough yet flexible and extensible. One good example of this type of cuticle is in ovipositing female locusts,

whose intersegmental arthrodial membranes expand 20-fold during oviposition (Gullan and Cranston, 2000). Where cuticle structures undergo spring-like elastic movement (*e.g.* the jump of a flea and at the base of wings), **rubber-like** cuticle is present (Gillott, 1995). A specialised protein, resilin, is present in this type of procuticle, and the coiled polypeptide chains produce the release of elastic energy (Gullan and Cranston, 2000)

The procuticle can be 10μm to 0.5mm thick and is composed almost entirely of chitin microfibers in a protein matrix. Chitin (Figure 1.3) is a nitrogenous polysaccharide composed mainly of β 1-4 linked residues of *N*-acetyl-D-glucosamine (Gillott, 1995). Chitin is very similar to cellulose, another important structural polysaccharide, and is grouped together into bundles to form microfibers. Extensive hydrogen bonding of adjacent chitin microfibers occurs to create tensile strength and the microfibers are embedded in a protein matrix. Groups of microfibers are arranged in a succession of overlying sheets. The microfibers in each plane are parallel to each other and each sheet may be orientated at a slightly different angle to the previous sheet, which creates a helicoidal arrangement of layers (Gillott, 1995).

Recent studies have revealed that the epidermis secreted more than a dozen major cuticular proteins. The properties of these proteins are determined by their amino acid composition. In general, endocuticular proteins are rich in hydrophobic amino acids with bulky side chains that allow for loose packing of the molecules (Gillott, 1995). The flexibility that results facilitates intrastadial growth soft-bodied insects.

Figure 1.3: The structure of chitin (reproduced from Gillott, 1995)



Acetylglucosamine **Acetylglucosamine** **Glucosamine**

In contrast, exocuticular proteins are composed of small, compact amino acids that confer hardness and stiffness in the exocuticle (Hepburn, 1985).

Andersen *et al.* (1995) observed pronounced similarities in the cuticular proteins of the locust species *Locusta migratoria* and S. *gregaria*. Pre-ecdysal cuticle proteins of *L.migratoria* are hydrophobic, suggesting high alanine, proline, glycine, tyrosine and valine content, whilst post-ecdysal cuticle proteins are more hydrophilic and contain more acidic amino acids, *e.g.* aspartic acid and glutamic acid (Andersen, 1995). Andersen *et al.* (1995) suggested that the main features of the structures of the cuticular proteins had been conserved over the course of evolution (Gillespie *et al.*, 1998). Andersen *et al* (1995) also suggested that the different mechanical properties shown by the various types of cuticle (*i.e.* solid, arthrodial and rubber-like procuticle) can be explained by the type of individual proteins present, together with the degree of sclerotisation evident.

1.3.3. Formation of the cuticle

Apolysis (the separation of the epidermal surface from the overlying cuticle at the time of ecdysal droplet secretion) occurs and leads to a new generation of plasma membrane plaques (dense structures which occur on the tips of microvilli of an epidermal cell) which then secrete the new outer epicuticle. Tiny patches of the new envelope immediately covering the plaques soon cover the complete epidermal surface (Locke, 1984, Gillott, 1995). The newly formed outer epicuticle is then tanned to protect the epidermal cell layer and new procuticle from moulting fluid that digests away the old cuticle at ecdysis.

Pores of 30Å diameter (Locke, 1966) in the outer epicuticle layer may allow for reabsorption of the digestive products prior to ecdysis, whilst restraining the passage of enzymes of the moulting fluid (Neville, 1975). Quinone tanning also gives the outer epicuticle hydrophobic properties, so that it can support surface lipid layers (*e.g.* the wax layer of the epicuticle). At ecdysis the outer epicuticle undergoes further tanning, which makes it exceedingly impermeable to water, ions and small molecules. This impermeability is further strengthened by the addition of more lipid to the surface of the outer epicuticle at ecdysis.

The next part of the cuticle to be formed is the inner epicuticle, which separates the outer epicuticle from the underlying procuticle. The inner epicuticle is also quinone-tanned and forms the main part of the epicuticle. As mentioned in section 1.6.2., it contains polyphenol oxidase which is important for wound repair in the cuticle.

After the outer and inner epicuticle layers are formed, channels appear across the epicuticle. These are often filaments of 5-10 nm in diameter and are most abundant in areas of wax secretion. According to Locke (1984) these filaments are a means for cuticular lipids to reach the surface of the cuticle, as they are stabilised to become lipophilic after the epicuticle has been stabilised.

The channels formed in the epicuticle can extend into the fibrous procuticle as pore canals (see below). The procuticle is the next layer to be secreted by the epidermal layer. The layers of the procuticle are secreted in order, beginning with the outermost and working inwards. The procuticle consists of chitin fibres embedded in

a protein matrix (Filshie, 1982). These fibres arise from the same plasma membrane that secreted the outer epicuticle at the beginning of the integument formation process.

Pore canals are formed at the same time as the procuticle and extend from the plasma membrane into the procuticle. They are cytoplasmic extensions of the epidermal cells (Wigglesworth, 1976, Gullan and Cranston, 2000), formed by the fusion of several wax channels which seem to function rather like a wick of a candle in transporting lipid-water liquid crystals to the cuticle surface (Gillott, 1995). When viewed in section, pore canals appear to have a central lipid filament surrounded by the chitin microfibers arranged in a helicoidal pattern, set in a protein matrix. Pore canals may be 100nm in diameter (Locke, 1984). Neville (1975) suggests that pore canals are involved with binding polyphenols to proteins, and possibly in wax synthesis as well as its transport. Pore canals do not form in rubber-like procuticle. High-density lipophorin, a major insect plasma protein, has been postulated as a reusable 'shuttle' for transport of lipids in haemolymph between tissues (Surholt *et al.*, 1991).

Further vertical structures are formed within the cuticle at this time, including sensory ducts (*e.g.* dermal glands), cuticular rods (in some cuticles, where chitin crystallites run perpendicular to the surface) and muscle attachment structures. Once the cuticle of a larval form of an insect has been completely secreted, however, it is only a matter of time before apolysis is repeated and the whole secretory process begins again. However, post-ecdysal addition to the outside often occurs to maintain its strength during inter-moult growth.

1.3.4. Repair of the integument

Three mechanisms of repair of any wounds to the integument have been proposed. These are described below.

1) The first line of repair involves tanning and wax secretion in the wound area. When the cuticle of an insect is wounded or abraded, prophenolase enzymes in the epicuticle are released. These cause the epidermis to release polyphenols (Ashida and Brey, 1995, Rowley, *et al.*, 1990, Bidochka *et al.*, 1989), which tan the wounded surface (Lai Fook, 1966). The surface exposed as a result of the wound becomes hydrophilic upon wounding and this is reversed through tanning so that the surface becomes hydrophobic. This hydrophobicity is also achieved through new wax secretions from the pore canals and wax channels (Wigglesworth, 1976). Wax blooms can sometimes form over the surface of the wounded area.

Locke (1984) discusses a 'cuticular compartment', an area within the body metabolic pool of the insect, controlled by the epidermis, which is not stabilised and where a reserve of cuticle components and molecules are stored. It also functions as a reaction vessel secreting and pinocytosing macromolecules for repair purposes. It is from this compartment that the materials for wound repair are drawn.

2) The second mechanism of repair to the damaged integument occurs if a wound penetrates the entire integument. Haemolymph nearest the wound coagulates, creating a haemolymph plug. Cuticular enzymes may tan this plug to increase hardness. Later, insect haemocyte cells collect in the area of injury and form a hard,

dark plug, which is externalised following restoration of epithelial continuity of the epidermal cuticle layer. Antibacterial activity due to a compound called cecropin (produced in fat body and the epithelial cells of the integument) was also detected in the matrix of the cuticle of a silkworm larva *Bombyx mori,* in the presence of live bacteria (Brey *et al.*, 1993).

3) The third mechanism for cuticle repair is ecdysis. This process allows complete renewal of the integument. Ecdysis, as well as allowing for intrastadial growth in insects, can also facilitate the removal of all abraded and therefore weakened surfaces of the cuticle. It also creates a digestive environment, which would prove fatal to any spores, or micro-organisms that may have penetrated a wound, before they reach the vulnerable epidermis.

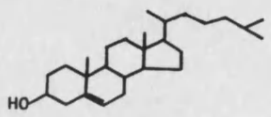
1.4.0. Insect epicuticular lipids

The cuticular lipids of insects are found in the outer layer of the integument (the wax layer of the epicuticle, see section 1.3.2.3.1 above) and consist of aliphatic material (Blomquist and Dillwith, 1985). Electron microscopy has also shown 'bound' lipid to be present within the epicuticle and procuticle, but very little is known about the chemistry of these inextractable lipids (Blomquist, 1984). In this section only the easily extractable surface lipids will be reviewed, as these have been extensively studied in recent years.

All insects studied contain hydrocarbons in their cuticular lipid profiles, and these are often the most abundant class of compound present (Blomquist and Dillwith, 1985, Lockey and Oraha, 1990, Howard, 1993). Polar compounds are also found in insect

cuticle extracts and these can include ketones, epoxides, primary and secondary alcohols, free fatty acids, free fatty acid esters, sterols, sterol esters and triacylglycerols (Oraha and Lockey, 1990). Figure 1.4 shows the structures of these common insect cuticular components.

Figure 1.4: Chemical structures of common insect cuticular components (reproduced from Blomquist and Dillwith, 1985)

n-alkanes	$CH_3-(CH_2)_x-CH_3$
n-alkenes	$CH_3-(CH_2)_x-CH=CH-(CH_2)_y-CH_3$
2-methylalkanes	$CH_3-\underset{\displaystyle CH_3}{CH}-(CH_2)_x-CH_3$
3-methylalkanes	$CH_3-CH_2-\underset{\displaystyle CH_3}{CH}-(CH_2)_y-CH_3$
internally branched monomethylakanes	$CH_3-(CH_2)_x-\underset{\displaystyle CH_3}{CH}-(CH_2)_y-CH_3$
dimethylalkanes	$CH_3-(CH_2)_x-\underset{\displaystyle CH_3}{CH}-(CH_2)_y-\underset{\displaystyle CH_3}{CH}-(CH_2)_z-CH_3$
trimethylalkanes	$CH_3-(CH_2)_y-[\underset{\displaystyle CH_3}{CH}-(CH_2)_y]_3-(CH_2)_z-CH_3$
primary alcohol wax esters	$CH_3-(CH_2)_x-O-\overset{\displaystyle O}{\overset{\|}{C}}-(CH_2)_y-CH_3$
secondary alcohol wax esters	$CH_3-(CH_2)_x-\underset{\displaystyle (CH_2)_y-CH_3}{\overset{\displaystyle H}{\overset{\|}{C}}-O-\overset{\displaystyle O}{\overset{\|}{C}}}-(CH_2)_z-CH_3$
epoxides	$CH_3-(CH_2)_x-\overset{\displaystyle O}{\overset{/\backslash}{CH-CH}}-(CH_2)_y-CH_3$
ketones	$CH_3-(CH_2)_x-\overset{\displaystyle O}{\overset{\|}{C}}-(CH_2)_y-CH_3$
secondary alcohols	$CH_3-(CH_2)_x-\underset{\displaystyle OH}{CH}-(CH_2)_y-CH_3$
primary alcohols	$CH_3-(CH_2)_x-CH_2-OH$
free fatty acids	$CH_3-(CH_2)_x-\overset{\displaystyle O}{\overset{\|}{C}}-OH$
sterols (cholesterol)	

The composition and thickness of the cuticular lipid layer varies intra-specifically, between sexes and between individuals. For example, in tobacco budworm pupae (*Heliothis virescens*), hydrocarbons constitute 0.5% of epicuticular lipid (Blomquist and Dillwith, 1985), in desert locusts (*S.gregaria*), hydrocarbons represent ~50-70% cuticular lipids (Lockey and Oraha, 1990), and in adults and nymphs of the field cricket, *Nemobius fasciatus*, 100% of cuticular lipids are hydrocarbons (Nelson, 1993).

1.4.1. Functions

Many of the functions of the insect integument described in section 1.3.1 can be directly attributed to the presence of epicuticular lipids. These include prevention of desiccation, an extreme example of this being scale insect species which possess a very thick layer of wax combined with cast skins. Cuticular lipids are also involved in chemical communication and hydrocarbons serve as sex pheromones, thermoregulatory pheromones and kairomones, (Blomquist and Dillwith, 1985). The lipoidal layer may also provide protection against penetration of pesticides, with the lipids acting as a barrier between the lipid soluble insecticides and the lower phases of the cuticle. Some short chain fatty acids within the lipid layer have also been linked with toxicity to pathogenic fungi (Koidsumi, 1957, Smith and Grula, 1982).

1.4.2. Extraction and separation

Cuticular lipids can be easily extracted from whole insects, cast exuviae or detached wings through immersion in organic solvents such a pentane, hexane and chloroform.

Methanol can also be used for lipid extraction but there is a danger of extracting internal lipids if extraction periods are prolonged (Blomquist and Dillwith, 1985). This possibility can be avoided, however, if detached insect wings are extracted or if immersion times are reduced.

Separation of cuticular lipids can be achieved by thin layer chromotography, gas-liquid chromotography (GLS) (Blomquist and Dillwith, 1985) and gas chromatography linked to mass spectrometry (GC-MS) (Oraha and Lockey, 1990 and Lockey and Oraha, 1990).

1.4.3. Non-polar lipids

The term 'hydrocarbon' is used here to describe the long-chain *n*-alkanes, alkenes and branched alkanes and alkenes (after Nelson, 1993) present in the cuticular lipids of insects. Hydrocarbons are those lipids present on the cuticle surface that can be extracted with non-polar organic solvents.

The biosynthesis and transport pathways of insect cuticular hydrocarbons have been well studied in many species (Blomquist 1984, Blomquist and Dillwith, 1985, Schal *et al.*, 1998). Biosynthetic pathways for hydrocarbons have been deduced for german cockroach nymphs, *Blatella germanica*, (Young and Schal, 1997, Schal *et al* 1998) the death's head hawk-moth, *Acherontia atropos* (Surholt *et al.*, 1991, 1992), the eggs of *Triatoma infestans*, a blood sucking bug (Juarez, 1994b), *Tenebrio molitor* (Blomquist, 1984) and the migratory locust, *Locusta migratoria*, (Surholt *et al.*, 1992) amongst many others. Lockey and Oraha (1990) have comprehensively documented their findings of the hydrocarbons of the desert locust, *S.gregaria*, as

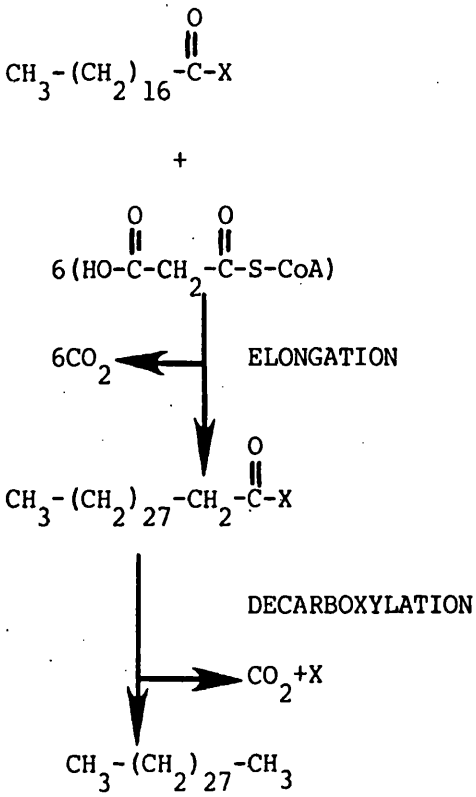
part of a larger study of the polar and non-polar cuticular lipids of adult migratory (*Locusta migratoria*) and desert locusts (Oraha and Lockey, 1990, Lockey and Oraha, 1990). Numerous reviews exist on occurrence, function and biosythesis of insect hydrocarbons, including Blomquist and Jackson (1979), Blomquist and Dillwith (1985), Lockey (1988) and Howard (1993).

Hydrocarbons common to insect cuticles include n-alkanes, alkenes and methyl-alkanes such as 2-methyl alkanes, 3-methyl alkanes and internally branched methylalkanes (Blomquist and Dillwith, 1985, Lockey and Oraha, 1990). All serve different purposes, with *n*-alkanes being mainly responsible for protection against desiccation (Gillott, 1995) and methyl branched alkanes serving as both pheromones and kairomones in some insects (Blomquist and Dillwith, 1985, Nelson, 1993).

The most likely sites of hydrocarbon biosynthesis are the oenocytes, which are situated near the integument and also around the peripheral regions of the fat body (Diehl, 1975, Gu *et al.*, 1995).

Hydrocarbons are formed by the microsomal elongation of fatty acids to form very long fatty acid chains, which are then reductively decarboxylated to *n*-alkanes, as illustrated in Figure 1.5 below. Any methyl branch units are added during the elongation stage (Blomquist, 1984). In alkene formation oleic acid specifically is elongated and decarboxylated (Blomquist and Jackson, 1979, Blomquist and Dillwith, 1985).

Figure 1.5: Biosynthesis of insect hydrocarbons (reproduced from Blomquist, 1984)



After synthesis the hydrocarbons are transported through the haemolymph by a specialised low-density lipoprotein known as lipophorin (Blomquist, 1984, Surholt *et al.*, 1992). Lipophorin has also been linked to lipid transportation of lipid energy to insect flight muscles (Surholt *et al.*, 1992). Lipids are transported to the cuticular surface via pore canals (see section 1.3.3 above).

Information obtained on cuticular hydrocarbons profiles has been utilised in studies on insect taxonomy and in investigations into chemical communication in insects (Blomquist and Dillwith, 1985, Howard, 1993). For example, hydrocarbon profiles have been used to distinguish between strains of the mosquito *Anopheles stephensii* (Anyanwu *et al.*, 1997) and between 3 North American species of *Blatella* cockroach (Carlson and Brenner, 1988). Gu and co-workers (1995) studied hydrocarbons as molecules of chemical communication by examining contact sex pheromones in the German cockroach (*Blatella germanica*). Udayagiri *et al.*, 1997, established that *n*-alkanes induced oviposition in *Ostrinia nubilalis.* Many other workers have investigated the role of cuticular hydrocarbons in chemical communcation with the view that this knowledge will be of use in insect control (reviewed in Blomquist and Dillwith, 1985 and Howard, 1993).

1.4.4. Polar lipids

In addition to hydrocarbons, many insect species possess more polar cuticular lipids with molecules containing one or more oxygen functional groups on long, aliphatic carbon chains (Buckner, 1993). Polar lipids common on the insect cuticular surfaces include wax esters, short chain fatty acid acid esters, ketones, epoxides, free fatty

acids, free primary alcohols, and sterols (Blomquist and Dillwith, 1985, Oraha and Lockey, 1990, Buckner, 1993). It is uncertain as to whether triacylglycerols are true components, as these could be contaminants from internal body lipids (Oraha and Lockey, 1990). Polar compounds have not been the subject of such extensive studies as non-polar cuticular lipids. However, from those insects studied, triacylglcerols, free fatty acids and free fatty acid esters predominate over wax esters (except in the production of beeswax, Blomquist and Dillwith, 1985).

In the desert locust, *S.gregaria*, esters are the most abundant polar compounds, comprising 6.7% of the cuticular lipid fraction (polar and non-polar) in females and 6.4% in males. Free fatty acids are present as 4.7% of the cuticular lipid (polar and non-polar) fraction in females, and 1.5% in males (Oraha and Lockey, 1990). Free primary alcohols can be present as 5.1% in female and 1.7 % in males. The cuticle of *S.gregaria* was found to lack sterols, free secondary alcohols and secondary alcohol esters, which can be found in the closely related grasshopper *Melonoplus sanguinipes* (Oraha and Lockey, 1990). These figures demonstrate the variation both between sexes and species, which exists in polar cuticular lipid composition.

It has been suggested that free primary alcohols in the cuticular lipids arise from the reduction of fatty acids, whilst secondary alcohols have been shown in grasshoppers to be formed through hydroxylation of the corresponding alkane. Both types of alcohols can be esterified to form wax esters (Blomquist, 1984, Blomquist and Dillwith, 1985). It is not known where, in or on the insect, these biosynthetic pathways occur.

The most significant of the relatively little work (when compared to that on hydrocarbons) conducted on polar cuticular lipid components has involved fatty acids. Koidsumi (1957) found compounds in the insect cuticular lipids had an anti-fungal effect, and went on to suggest that these compounds were short chain fatty acids. This was supported by the work of Smith and Grula (1982), and Barnes and Moore, (1997) who identified caprylic acid and fatty acids below C10 respectively, as antifungal agents. Polar compounds have proven more difficult to extract from the insect cuticle and occur in much smaller quantities, hence investigations into their function in the cuticle are scarce. However, with such indications as the above investigators have shown, the need for further research into these cuticular lipid components is evident.

1.5.0. The pathogen

1.5.1. *Metarhizium anisopliae* var. *acridum*

The most virulent of all fungal pathogens of acridoid species are from the genus *Metarhizium*. The genus has a world-wide distribution and includes three species, *Metarhizium anisopliae*, *Metarhizium flavoviride* and *Metarhizium album* (Bridge *et al.*, 1993). Most variants were originally isolated from soil and commonly infect soil dwelling Coleopterans Liu *et al.*, 1989). Variants and isolates of *M.anisopliae* have been used against a wide variety of host insect pests across the world. Research into the host range of *M.anisopliae* in particular has revealed that different isolates can infect over 200 known hosts covering insect orders such as Orthoptera, Hemiptera and Dictyoptera as well as Coleoptera (Bateman *et al.*, 1993, Butt *et al.*, 1995). *M. anisopliae* species are commonly called 'green muscardine' because of the green colour of the sporulating mycelium on insect cadavers (Bartlett and Jaronski, 1988).

Mass production of this species by Metchnikoff in 1880, and by Krassilstchik in 1884 marked the first use of artificial substrates to grow entomopathogenic fungi (Bartlett and Jaronski, 1988). These researchers achieved 180-220g conidia per $1m^3$ medium in 15 days. *M.anisopliae* was virtually ignored as a control method, however, until the 1960's, a number of small manufacturers in Brazil began production of Metaquino™, for control of insect pests. Mass production was facilitated by growth of the fungus on autoclaved rice or wheat bran (Bartlett and Jaronski, 1988). Since then, mass production of the fungus has occurred in the USSR, Australia and New Zealand (Bartlett and Jaronski, 1988), among many other countries.

The most virulent and host-specific variant of the species to locusts and grasshoppers is *M.anisopliae* var. *acridum*. Isolates of *M.anisopliae* var. *acridum* were formerly placed in the species *Metarhizium flavoviride*, but their membership of the species *M.anisopliae* has recently been established (Driver *et al.*, 2000).

M.anisopliae var. *acridum* was isolated from a grasshopper, *Ornithacris cavroisi* (Finot) (Orthoptera: Acrididae) in Naimey, Niger, 1988 (Bridge *et al.*, 1993, Stathers, *et.al.*, 1993, Moore *et.al.*, 1995) and the isolate IMI 330189 has become the standard isolate for both field and laboratory trials of locust pests such as *S. gregaria* and *Locusta migratoria migratorioides*. As previously noted, the isolate has also recently been registered in South Africa as Green Muscle™ for use against the brown locust, *Locustana pardalina* (Bateman *et al.*, Green Muscle® User Handbook, 1998). It is anticipated that commercial use will eventually also occur in Africa (against locusts

and grasshoppers) and Australia (against the plague locust, *Chortoicetes terminifera*) (Moore, pers. comm.).

1.5.2. The infection cycle of entomopathogenic fungi

Metarhizium anisopliae var. *acridum* and other entomopathogenic fungi, initiate mycosis through an infective unit called the conidium, although some genera utilise zoospores. The first event of mycosis to occur is the adhesion of the conidium to its host. Interactions which allow attachment of an infective structure to its host can be specific and active (*e.g. Lagenidium giganteum* zoospores are host selective and employ chemotaxis, Kerwin, 1986) or non-specific and passive (*e.g. M.anisopliae* and *B.bassiana,* Charnley, 1989a).

Conidial adhesion is the very first step of mycosis for entomopathogenic fungi. Fargues (1984) divided fungal adhesion into three stages:

(1) the absorption of spores to the cuticle – where charged groups on the spore and host surfaces initiate attraction

(2) attachment – where short-range stereochemical interactions, involving surface antibodies, lectins, glycoproteins and sugars occur

(3) germination and growth of the fungus on the surface of its host prior to penetration peg formation – where nutrients, such as lipids, sugars and amino acids, on the host surface may alter the attachment from a 'passive' to an 'active' process by the induction of enzymes and mucilage from germinating conidia, germ tubes or appressoria.

Attachment is mediated by strong, yet non-specific binding forces in hydrophobic conidia of insect-pathogenic fungi such as *B.bassiana*, *N.rileyi* and *M.anisopliae.* Hydrophobicity of the conidial wall in these fungi is believed to mediate the adhesion process (Boucias *et al.*, 1988). The outer topography of the conidia is thought to play a major part in the adhesion process. The outer surface of the dry conidia is made up of well organised, resilient, interwoven fascicles of rodlets (Boucias *et al.,* 1988).

Physical properties of the surface of the host itself, such as hydrophobicity, also affect the adhesion of fungal spores. The more hydrophobic the surface, the more tenacious the adhesion (Terhune and Hoch, 1993).

Some entomopathogenic fungi, such as those within the genus *Entomophthora* achieve adhesion via hydrophilic conidia. These conidia lack the rodlet arrangement, but are covered in a layer of mucilaginous material (Boucias and Pendland, 1991) which acts as an adhesive. Other entomogenous fungi attach to the cuticle of their hosts by the encystment of motile zoospores. This is demonstrated by the water mold species such as *Lagenidium* spp. Once the flagellum has been retracted, an amorphous substance is secreted onto the integument of the host. The adhesive material is thought to consist of glycoproteins (Boucias and Pendland, 1991). A cyst wall is then laid down, from which further infection processes, such as germ tube formation, occur.

The chemistry of the insect cuticle itself can affect conidial adhesion (Sosa-Gomez *et al.*, 1997). The role of cuticular lipids must be taken into account when relating

fungal adhesion to the chemical nature of cuticular surfaces (Fargues, 1984). Attachment of conidia to the cuticle of an insect is dependent on the chemical interactions between the epicuticular lipid layer (the 'wax layer' described in section above) and surface of the conidia (Boucias and Pendland, 1984a). Indeed, the wax layer has been documented as containing anti-microbial compounds and may be responsible for preventing germination of some potentially entomopathogenic fungi (Boucias and Pendland, 1984a). For example, Koidsumi (1957) found free, straight-chain, saturated fatty acids in lepidopteran cuticular ether extracts inhibited germination of *B.bassiana* and Smith and Grula (1982) demonstrated that medium-length fatty acids inhibited germination of *B.bassiana* on *Heliothis zea*. The removal of surface lipids with organic solvents (*e.g.* ether and chloroform) (Sosa-Gomez *et al.*, 1997) or through physical abrasion (Blomquist and Dillwith, 1985) has also been shown to increase susceptibility of insects to entomopathogenic fungi (Fargues, 1984).

Some cuticular lipids are utilised purely as a binding substrate by entomopathogenic fungi, (*e.g.* *n*C21 to *n*C31 hydrocarbons found on *Nezara viridula,* stink bug, cuticle) whilst other lipids play a very important nutritional role in stimulation and subsequent growth of the conidial germ tube (Boucias and Pendland, 1984a).

Further polar components extracted with methanol have also been found to stimulate a conidium of *M.anisopliae* during the stages of adhesion, on the surface of the scarabeid larvae (Fargues, 1984). These polar components included salts, amino acids and proteins.

The topography of the insect epicuticle surface also plays a role in attachment. Sosa-Gomez *et al.* (1997) found that conidia became lodged in areas of the cuticle with large numbers of setae (*e.g.* antennal tips, the apical portions of tarsae). Distribution of conidia can also be affected by intersegmental folds and surface roughness (Fargues, 1984), which could create beneficial pockets of high humidity on the cuticle.

The microflora present on the surface of an insect can be either stimulatory, as shown by Fargues (1984), where *M.anisopliae* conidia germinted to higher levels in the presence of contaminants, or detrimental, and lead to antibiosis (Schabel, 1976). Enzymes on the surface of the conidium may modify host cuticle and improve adhesion. Once a conidium has formed an attachment, the next step in the invasion process, germination, may begin.

Conidial germination usually occurs in areas of localised high humidity such as the spiracles, mouthparts, intersegmental spaces, and arthrodial membranes, all of which are made from non-sclerotised cuticle, which is easier to penetrate (Hajek and St Leger, 1994). Germination is dependent on a number of factors besides moisture availability, such as temperature, a suitable nutrient source (Gillespie and Moorhouse, 1989) and the presence of other micro-organisms on the cuticle which may inhibit the process (Schabel, 1976).

Nutrient-insufficient conidia, such as those from *M.anisopliae* and *B.bassiana*, require an exogenous source of carbon and nitrogen to initiate germination. Absorption then occurs, leading to spherical growth (swelling) and germ tube

production. Nutritional requirements for germination are more specific for those entomopathogens of restricted host range, whilst entomopathogens with broader host ranges usually have less fastidious nutrient requirements for germination (Charnley, 1989a). Nevertheless, nutrient availability can still prove limiting on the latter pathogens (Grula *et al.*, 1978).

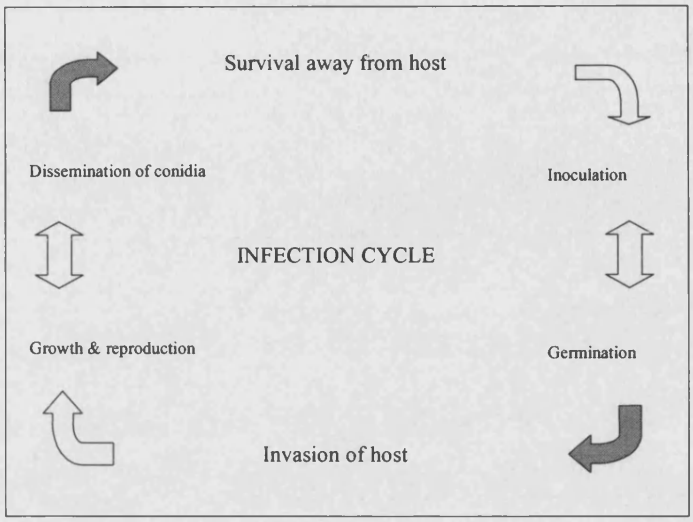
After germination, the fungus may penetrate the cuticle of a host insect directly, as in *V.lecanii* (Hughes and Gillespie, 1985) or by means of a penetration peg produced by an infection structure or appressorium (Zackaruk, 1970). Penetration of the insect cuticle results from a combination of mechanical pressure and enzymic degradation of the cuticle (using protease, lipase and chitinase enzymes). An extensive study by St Leger and co-workers has established in particular the role of endoproteases in this process (St. Leger *et. al.*, 1986a and c). As invasion of a host occurs directly through the exoskeleton, insect pathogenic fungi are unique among entomopathogens in that they can attack non-feeding stages such as pupae and eggs (Charnley, 1997).

Once a penetrant hypha breaks through the cuticle and underlying epidermis it enters the haemocoel of the insect. Some fungi, such as *Entomophthora coronata* in the termite *Reticulitermes flavipes* (Yendol and Paschke, 1965) continue filamentous growth as hyphae, whereas most change to yeast-like bodies. These so-called blastspores proliferate by budding and circulate in the haemolymph (Roberts and Humber, 1981), and in the Deuteromycotina, the fungus has a short parasitic existence within the insect (Charnley, 1989b).

Host death can be due to the autointoxication brought on by extensive mechanical damage after extensive hyphal growth in the insect's tissues and haemolymph and inappropriate release of reactive host molecules (Charnley, 1989a), or by depletion of nutrient resources during the pathogen's parasitic phase (Charnley, 1989b). In the Deuteromycotina, the low molecular weight cytotoxins, e.g. destruxins produced by *M.anisopliae* var. *acridum* may promote disease development and hasten death (Charnley, 1989b, Charnley, 1997).

After host death the entomopathogenic fungus reverts to mycelial growth and invades virtually all organs of the host. The cadaver can often serve as a reservoir of fungus during periods of adverse conditions (Roberts and Humber, 1981). Colonisation of the cadaver, marks the beginning of a phase of saprophytic growth for the fungus, and providing there is adequate moisture, the hyphae penetrate to the outside surface of the cuticle. The fungus emerges at areas that pose less resistance such as intersegmental and arthrodial membranes, and sporulation occurs on the exterior of the cadaver (Gillespie and Moorhouse, 1989). The final stage of the infection cycle is the dispersal of the infective units (usually conidia) to areas where they will come into contact with further suitable hosts. This can be achieved passively, as in the Deuteromycotina, through wind and water, or forcefully, as with the Entomophthorales, which discharge their spores (Roberts and Humber, 1981). The infection cycle is summarised below in Figure 1.6.

Figure 1.6: Schematic representation of the entomopathogen infection cycle



1.6.0. Fungal nutrition and metabolism

As outlined in section 1.5.2 above, exogenous and endogenous nutrients are vital triggers to germination of conidia of entomopathogenic fungi. Some fungi, such as plant pathogenic rusts (*e.g. Neurospora tetrasperma*) possess spores with an abundance of stored nutrients, which can germinate independently of nutrients present on the surface of their hosts (Weete, 1981). Other fungi, such as the entomopathogens *B.bassiana* and *M.anisopliae*, are nutrient-dependent, and germination is consequent upon an exogenous nutrient source (Charnley, 1997). The latter are focussed on in this review.

Nutrient-insufficient conidia rely on non-specific accessible sources of nitrogen and carbon for germination *in vitro*. For example, *B.bassiana* can grow copiously on a simple carbon source such as glucose, to a complex wax (Woods and Grula, 1984).

In vivo, however, more selective pathogens are thought to have more specific nutritional requirements (Charnley, 1997), in that they may respond to qualitative and quantitative nutrient availability on their hosts' surface. The two most important nutrient sources for fungi, nitrogen and carbon, and their metabolism in fungi, are described below.

1.6.1. Carbon

Carbon sources utilised by fungi occur in the form of monosaccharides, disaccharides and hydrocarbons.

Almost invariably, the monosaccharide glucose is one of the sugars most readily utilised by nearly all fungi. This is supported by the detection of glycolysis and the pentose phosphate pathway in virtually all fungi except for *S.cerevisiae.* In filamentous fungi such as *Neurospora crassa,* uptake of monosaccharides occurs by facilitated diffusion (Jennings, 1995).

Disaccharides may be transported intact across the plasma membrane of fungi, or may first be hydrolysed externally and the products then actively transported into the cell. It is thought that these two systems are not mutually exclusive (Jennings, 1995).

Aliphatic hydrocarbons have been found in almost every plant, animal and micro-organism examined (Reisener, 1976). Hydrocarbons, particularly *n*-alkanes and methyl branched alkanes, are also the most common and abundant class of lipid reported in insect cuticle, constituting 76% of total lipid extracted from the cuticle of the desert locust, *Schistocerca gregaria* (Lockey, 1976). *M.anisopliae* and

Nomuraea rileyi can utilise *n*-alkanes as the sole source of carbon (St Leger *et al.*, 1988a).

In the yeast *Candida tropicans*, very little alkane is taken into the cell in solution. Instead, the cells adhere to alkane droplets. Submicrometre droplets of various sizes can adhere to the cell walls and the affinity of the cell wall for alkanes has been shown to be independent of pH, temperature and chain length of the alkanes (Jennings, 1995). St. Leger *et al* (1988a) suggested that contact between hyphae and an insoluble hydrocarbon may be a prerequisite for uptake. Adhesion of a fungal cell to alkane droplets may be promoted by the production of a surface-localised mannan-fatty acid complex. The production of hair-like structures on the cell surface, the production of lipoproteins, glycolipids and lipopolysaccharides (emulsifying agents) to reduce interfacial tension of the liquid droplets or the production of extracellular sphorolipids to make the fungal wall more lipophilic for better contact with the alkane droplets may also aid this process (Gareth-Jones, 1994). It is not known which, of the systems, if any, is utilised by entomopathogenic fungi to facilitate transport of alkanes into its cells.

Once inside the fungal cell alkanes are oxidised to fatty acids. Although the exact process is uncertain, three possible pathways are suggested by Jennings (1995):
(1) Hydroxylation by a monooxidase to form a primary alcohol, (2) Dehydrogenation to the corresponding alkenes and (3) Incorporation of oxygen into the alkanes by a dioxygenase to produce an n-alkyl hydroperoxide, which in turn is reduced to a primary alcohol (hyperperoxidation). Primary alcohols in all cases are then oxidised to the corresponding fatty acids through an aldehyde dehydrogenase. In yeasts, the

above processes have been found to involve peroxisomes, mitochondria and microsomes (Jennings, 1995) although the same has yet to be proved in higher fungi.

Other lipids found in fungal spores include fatty acids, glycerides, phospholipids, glycolipids, sterols and carotenoids. These lipid components have been observed as the chief storage metabolites and the primary substrates used during germination of fungal spores (Reisener, 1976). The most important of these additional lipids to fungi are the fatty acids. Fatty acids are taken up, depending on their chain length (different species being selective for different fatty acids) into fungal spores during germination, and oxidised during the germination process. In rust fungi short chain even numbered fatty acids are oxidised to form acetylCoA only, whilst short chain odd-numbered fatty acids produce a terminal propionylCoA residue (Reisener, 1976). Both of these compounds are important precursors in fungal respiration in catabolic pathways. Longer chain fatty acids were oxidised in the same way in further studies. However, these data on rust fungi uredospore germination cannot be confidently extended to other species of fungi. Furthermore, a shift from one substrate to another can occur during the germination of one spore, so generalisations cannot be relied upon (Reisener, 1976).

1.6.2. Nitrogen

Species of fungi can vary in the source of nitrogen they can utilise, including nitrate, nitrite, ammonia and a very large range of organic nitrogen compounds. No known fungus has been shown yet to be capable of fixing atmospheric nitrogen (Jennings, 1995). Some fungi, such as parasitic fungi like the rust *Puccinia graminis* and lower fungi, cannot germinate or grow when supplied with ammonia as a sole nitrogen

source, but require sulphur-containing amino acids (Jennings, 1995). On the other hand, fungi such a *Rhizopus oryzae* require ammonia for germination and growth (Jennings 1995).

Amino acids, peptides and proteins are present on the surface of insect cuticle (Woods and Grula, 1984 and Chapter 3. of this thesis). They have been shown to be present in sufficient quantities on the surface of the corn earworm, *Heliothis zea* to support germination and limited growth of entomopathogenic fungi such as *B. bassiana in vitro* (Woods and Grula, 1984). *M.anisopliae* produces several families of endo- and exo- acting protease that can combine to hydrolyse cuticular peptides and proteins (Clarkson and Charnley, 1996).

In general, it has been found that peptides can be transported into the fungal cell intact, by a peptide transport system. Once inside the cell, peptides are hydrolysed by intracellular peptidase enzymes to release amino acids (Jennings, 1995). Free amino acids on the surface are transported inside fungal cells by a number of genetically and morphologically distinct transport systems. In higher fungi transport systems exist for (I) neutral and aromatic amino acids, (II) general transport of neutral, basic and acidic amino acids, (III) basic amino acids (IV) acidic amino acids and (V) methionine. These systems are used under different nutrient conditions (*e.g.* carbon, nitrogen or sulphur starvation) in both conidia and mycelia.

1.7.0. Aims of this study

The conidia of the entomopathogenic fungus *Metarhizium anisopliae* var. *acridum* are nutrient insufficient, relying on nutrients and signals on the surface of the host insect to initiate the first stages of pathogenesis. This could be a causative factor of the slow speed of kill observed in the field. The aims of this work were to investigate the behaviour of the fungus during important initial infection events (adhesion, germination and appressoria formation), using *in vitro* and semi-*in vivo* techniques, and to examine the interactions between the fungus and nutrients/signals on the surface of the host. Solvent extractions, glucose and protein assays, GC-MS, TLC, Cryo-SEM and Atomic Force Microscopy were among the methods used to gain insight into these interactions.

Previous studies on the role of epicuticular lipids have been limited in scope or technically flawed. For example, Lecuona *et al* (1991) extracted insects 96 h after inoculation with *Beauveria* spp. but could not distinguish insect from fungal lipids. The laminarinase removal method developed as part of the present work does, however, facilitate this distinction.

The present work also looked into the specificity of *M.anisopliae* var. *acridum*, with the aim of establishing as to whether the basis of such high level specificity lay at the level of host epicuticular components. Specificity was tested with bioassays using blowfly and flour beetle wings.

2 Materials and Methods

All chemicals and enzymes, unless otherwise stated, were obtained from Sigma-Aldrich Ltd. chemical company. Ingredients for media were supplied by LabM Ltd. and BDH Ltd.

2.1.0 Maintenance of fungal and insect cultures

2.1.1 Culturing the fungus

Cultures of the fungus ***Metarhizium anisopliae* var. *acridum*,** isolate **IMI 330189,** were maintained from stock conidial suspensions of 1 x 10^7 conidia ml^{-1}, stored at -70°C in 10% glycerol. At 2-3 month intervals, a stock suspension was removed from storage, defrosted and inoculated onto plates containing ¼ strength Sabouraud's Dextrose Agar (SDA) plates (see Appendix for recipe). These plates were cultured at 27°C and at 100% humidity for 10-14 days, and stored thereafter at 4°C.

Conidial suspensions were made from 14 day old growing cultures by washing with sterilised dH_2O or 0.04% Tween 80 through a filter of 2 layers of sterile muslin. Spore suspensions were then washed twice by centrifugation, the supernatant was decanted, and the spore pellet was re-suspended in the appropriate medium. Full aseptic technique was used throughout harvesting, and conidial suspensions in sterile dH_2O were sonicated for 20 minutes prior to use, to avoid clumping of conidia.

The concentration of conidia within suspensions was determined using a haemocytometer (Weber Scientific International) and appropriate dilutions made.

Spore suspensions were tested for viability before each experiment using a technique adapted from James (1995). ¼ strength SDA was spread into 3 small pools on a glass microscope slide using a Pasteur pipette. 0.01ml of the spore suspension, diluted with dH_2O to 0.5 x 10^6 conidia ml^{-1} was introduced onto each pool of agar. The slide was left to dry for 5 min and then placed onto a piece of Whatman No.1 filter paper lining the lid of a 9cm non-vented petri dish (Sterilin®). The base of the petri dish was then placed on top of the lid and the preparation was incubated at 27°C at 100% humidity. Germination was assessed between 12 and 24 h after inoculation. Germination was judged to have occurred when a germ tube from a conidium was as long as it was broad (Manners, 1966). Assessment of germination was carried out by counting 100 conidia per pool under the light microscope, with viabilty being expressed as as the mean germination percentage of 300 conidia. Conidial suspensions that reached greater than 95% germination were used.

2.1.2 Insect cultures

Cultures of the desert locust, ***Schistocerca gregaria*** (Forskål) (Orthoptera: Acrididae) were maintained by the Department of Biology and Biochemistry insectary, at the University of Bath. Male and female of the species are shown in figure 2.1. Rearing conditions included a room temperature of 28°C, cage temperature of 32-36°C, and a 16 h day light/dark cycle. Diet for the culture consisted of bran with dried yeast sprayed periodically with a solution of sodium sulfamethazine (4.26% w/v), sodium sulfathiazole (3.65% w/v) and sodium

sulfamerazine (3.13% w/v) for control of the parasite *Malamoeba locustae*. Fresh wheat and water (treated periodically with a 5% solution of the above triple sulphur solution) were also provided. Eggs were incubated at 34°C.

Figure 2.1: Adult female (left) and male (right) desert locust, *Schistocerca gregaria*

2.2.0 The fungus and what it can do

2.2.1 Germination on ¼ SDA *in vitro*

Plates containing ¼ strength SDA were inoculated with a 1 x 10^6 conidia ml^{-1} in dH_2O suspension, using the free-standing Potter tower spray apparatus. This sprayer functioned by dispersing the liquid inoculum (via pressurised nitrogen gas) down a vertical stainless steel tube that enclosed the plate. In this way the Potter tower

apparatus facilitated a uniform coverage of the plate surface with conidia. Plates were then placed in a humid environment in an incubation chamber (Gallenkamp) at 27°C. Germination was assessed every 2 hours from 10 to 16 hours post inoculation. A glass cover slip was placed on a section of the plate and this section was examined under the light microscope. 5 separate sections of each of 5 plates were examined at each time interval and 300 conidia were counted for each section, so that 1500 spores were counted and assessed for germination for each plate. Germination at each time interval post inoculation was expressed as a percentage.

2.2.2 Appressoria formation *in vitro*

Appressorium formation was assessed using a similar procedure to that described above. ¼ strength SDA plates were inoculated with 1 x 10^6 conidia ml^{-1} dH_2O spore suspension and incubated at 27°C at 100% humidity. Counts were taken every 2 h for 6 h after appressoria were first observed (usually at ~20 h post inoculation for *M.anisopliae* var. *acridum*). Five sections on each of 5 plates were observed under the microscope and the number of appressoria per 300 conidia in each section of the plate noted. An appressorium was defined as an apical swelling on the germ tube of similar size to a conidium.

2.2.3 Germination in 0.04% Tween 80 *vs.* dH_2O

Two conidial suspensions were prepared, one harvested through a muslin filter in 0.04% Tween 80 and the other harvested through a muslin filter in sterilised dH_2O. Both solutions were sonicated for 20 min before use. Conidia were also washed twice to remove any trace of ¼ SDA which could be utilised to aid germination, by centrifugation of the conidial suspensions, decanting the supernatant and re-

suspending the conidia pellet in the appropriate medium (0.04% Tween 80 or sterilised dH_2O). In order to test the ability of conidia to germinate in the harvesting medium, 250µl of a very dilute conidia suspension of ~400 conidia ml^{-1} was placed in each well of a Sero-Wel® (Bibby Sterilin Ltd) microtitre plate, using aseptic precautions. A separate plate was prepared for the 0.04% Tween 80 and for the sterilised dH_2O solutions. After incubation at 27°C and at 100% humidity for 16 h, 100 conidia from each well of the microtitre plate were counted, using an inverse microscope (Nikon, TMS-F) and the number germinated was recorded, for each preparation. The number germinated was expressed as a mean of all the wells for each microtitre plate, for each harvesting medium tested.

2.2.4 Germination in Ondina El oil

Conidia were harvested in 10ml Ondina El oil (the formulation oil used in commercial preparations of *M.anisopliae* var. *acridum*, Green Muscle™, Bateman *et al.*, Green Muscle User Handbook, 1998), which was introduced directly onto the fungal culture growing on the surface of a ¼ strength SDA plate. The suspension was aliquoted into a number of 1.5ml eppendorf tubes. After washing by centrifugation as described above, the solution was diluted further with Ondina El oil and a small drop placed in the central well of a haemocytometer in order to determine the concentration of the conidial suspension. Once a concentration of 4 x 10^6 conidia ml^{-1} was achieved, the suspension was sonicated for 20 min to reduce clumping of spores.

0.5ml of the conidial suspension was then inoculated onto 2% water agar plates (a medium which had in previous experiments been shown to support negligible

germination of *M.anisopliae* var. *acridum*) using the Badger™ spray apparatus, which consists of a spray nozzle attached to a pressurised container. 2 control treatments were also prepared, where the conidia were suspended in sterilised dH_2O and sprayed onto 2% water agar plates and onto ¼ strength SDA plates.

After inoculation the plates were incubated at 27°C in conditions of 100% humidity for time intervals of 24, 30 and 48 h, and germination counts were made for each treatment at these times. Counts were made according to the procedure outlined in section 2.2.1.

2.2.5 Sterilisation of desert locust hind wings

Unless otherwise specified, all desert locust hind wings used for bioassay experiments were sterilised immediately upon detachment from live desert locust (10 days after ecdysis) adults and used immediately after sterilisation. Excised locust hind wings were placed in the lid of a glass petri dish, whilst the base was placed adjacent to the lid and filled with 25 ml propylene oxide (Fluka Ltd.). This preparation was then sealed inside a polythene bag and left in a fume cupboard overnight. Desert locust hind wings were selected as an ideal material for studying germination and appressoria formation of *M.anisopliae* var. *acridum* because their epicuticular composition (Oraha and Lockey, 1990) is essentially similar to that of the body cuticle, but with the advantages of being transparent (advantageous for examination under the light microscope) and of avoiding any contamination from other desert locust body lipids.

2.2.6 Inoculation and incubation of desert locust hind wings

The following procedure was used for all experiments where desert locust hind wings were inoculated with *M.anisopliae* var. *acridum*. 2-3 sterilised desert locust hind wings were spread flat on 2% water agar plates (see Appendix for recipe) and inoculated with a 1 x 10^6 conidia ml^{-1} in dH_2O suspension, using the Potter tower free-standing spray equipment, which facilitated more uniform coverage of the plate and wings with conidia than the Badger™ apparatus.

Germination and appressoria formation characteristics of *M.anisopliae* var. *acidum* were investigated for the desert locust hind wing. Newly excised hind wings placed on 2% water agar plates (figure 2.2) were inoculated with conidia as described above, and incubated at 27°C at 100% humidity (plates were enclosed in humid chambers within an upright incubator). Germination and appressoria counts were made by careful removal of a flat piece of wing from the plate. The piece of wing was mounted on a microscope slide and fungus stained with a small drop of 0.1% cotton blue in lactophenol. A cover slip was applied and the wing piece was then observed under the light microscope. Counts of germination and appressoria formation were made using the procedure outlined in parts 2.2.1 and 2.2.2 of this section respectively. Germination counts were taken at 6, 8, 10 and 12 h post inoculation, and appressoria counts were made at 12, 18, 24 and 30 h after inoculation. Experiments were conducted on sterilised wings as a standard preparation, and compared with non-sterilised hind wings and with hind wings excised from desert locusts reared under axenic (germ free) conditions. The differences in germination numbers at 12 h of the three wing treatments and appressoria formation at 30 h of *M.anisopliae* var. *acridum* post inoculation for each

of the three wing treatments were compared using a non-parametric test which compares the medians of more than two samples, known as the Kruskal-Wallis test. Full details of this test are given in the Appendices. Germination at 12 h on sterilised desert locust hind wings (standard preparation) was compared to germination on ¼ strength SDA, using the Mann-Whitney U statistical test, a non-parametric technique for comparing the medians of two unmatched samples. The Mann-Whitney U test was also used to compare the appressoria formation of *M.anisopliae* var. *acridum* to that on ¼ SDA medium. Full details are given within the Appendices.

Figure 2.2: Locust hind wings on 2% water agar plate

2.2.7 Germination of *M.anisopliae* var. *acridum* on hind wings taken from insects from different insect Orders

Wings from the Dipteran *Calliphora vomitoria* (blowfly) and the Coleopteran *Tenebrio molitor* (flour beetle) were excised and sterilised in an atmosphere of propylene oxide. They were then spread on 2% water agar plates, inoculated with 1 x 10^6 conidia ml^{-1} suspension, harvested in sterilised dH_2O and incubated at 27°C at 100% humidity. Control preparations were included in the study – these were 2% water agar plates inoculated with conidia and plates containing untreated desert locust hind wings.

Germination was assessed at 18 and 24 h after inoculation. 100 conidia were counted per wing instead of the 300 counted in locust experiments due to the small size of the fly and beetle wings. 15 wings were examined per treatment and there were 5 replicates. Germination at each time interval were expressed as a percentage.

The experiment was repeated in order to assess appressoria formation per 100 germinated conidia at 18, 24 and 30 h post inoculation.

The Kruskal Wallis statistical test was employed to compare germination on *C.vomitoria* wings and germination on *T.molitor* wings with germination on the standard preparation of sterilised desert locust hind wings. Appressoria formation on C.vomitoria and *T.molitor* was also compared to that on the standard desert locust hind wing preparation using the Kruskal Wallis test (see Appendices).

2.3.0 Investigation into desert locust hind wing epicuticular components

2.3.1 Extraction procedure

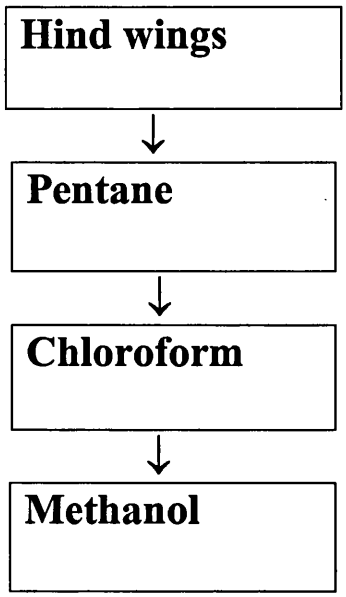
The following procedure was developed to extract ALL lipid materials (polar and non-polar) present on the epicuticular surface of the desert locust hind wing. It was developed in collaboration with Prof. M. Sainsbury, Department of Chemistry, University of Bath, UK. All solvents used were HPLC grade.

Excised locust wings were removed from adult desert locusts 10 days after ecdysis and immediately sterilised, as outlined in part 2.2.5 of this section. 30 of these hind wings were then placed in a Quickfit® acid-washed, round bottom flask. The first solvent used for extraction was re-distilled pentane (for removal of any hydrocarbons from the wings), and 30ml was poured into the flask. The flask was then attached to a Quickfit® reflux condenser apparatus and placed over a heating unit with a clamp and stand. A cooling water supply was connected to the reflux condenser and the contents of the flask heated to boiling point for an hour. The entire apparatus was enclosed within a fume cupboard to avoid exposure to the solvents used.

This procedure was repeated using chloroform and then methanol solvents, to remove any polar components which may have been present in the epicuticle (see Figure 2.3 for diagram of extraction procedure). Wings were washed 3 times with each solvent, before being treated with the next solvent, to ensure that all possible lipids were removed by a specific solvent, *ie.* to ensure that no soluble residues remained after refluxing the wings, which might contaminate the extract made with the next solvent in the series. Clean, acid washed glassware was used with each different solvent.

After hind wings had been removed, the extract for each solvent treatment was transferred into airtight 2ml glass vials (HPLC Technology Ltd.) and placed in an Edwards vacuum freeze drying apparatus (Modulyo Ltd.) to remove the solvent. The remaining extract was then re-suspended in 1ml of the original solvent and stored under nitrogen at -70°C, to await analysis.

Figure 2.3: Sequence of extraction of desert locust hind wings



2.3.2 Germination of *M.anisopliae* var. *acridum* on extracted locust hind wings

Wings extracted in pentane were spread flat (3 wings per plate) on the surface of 2% water agar plates and inoculated with 1 x 10^6 conidia ml^{-1} in sterilised dH_2O. This was repeated for wings extracted with chloroform and methanol. 3 plates were prepared for each solvent treatment, containing 3 wings per plate. Control plates of locust wings which had not been treated with any solvents (but had been sterilised with propylene oxide) and of 2% water agar plates alone were also inoculated. The treatments were incubated at 100% humidity and at 27°C and germination determined (per 300 conidia per wing piece) at 12, 18 and 24 h post inoculation.

Appressoria formation on extracted wings was also determined per 300 conidia for wings of different treatments. These assessments were made at 18 and 24 h after inoculation.

The Kruskal-Wallis test was used to statistically compare the extracted wing germination and appressoria formation data (see Appendices for full details of the test).

2.4.0 Analysis of hind wing before and after fungal attack

2.4.1 Development of technique for enzymic removal of germinated fungus from hind wing surface

A protocol was designed to remove germinating *M.anisopliae* var. *acridum* conidia from the surface of glass slides and, ultimately from the surface of desert locust hind wings. This protocol enabled measurement of epicuticular lipids before and after fungal attack without contamination from fungal lipids.

A combination of physical and chemical means was employed to remove germinating *M.anisopliae* var. *acridum* spores. A controlled water was jet created using a water pump attached to a water bath. The water bath was filled with sterilised dH_2O and 6.0mm-diameter rubber tubing attached to the water pump. The sample to be treated was held 30 cm from the rubber tubing and subjected to the jet of water for 20 sec.

Enzymic hydrolysis provided the chemical means for removal of the fungus. The enzyme laminarinase (from *Trichoderma* spp.) hydrolyses β 1,3 glucans, which appear to be the primary constituent of the mucus adhesive produced by *M.anisopliae* var. *acridum*, and was used, at its optimal pH of 5.0 (in McIlvaine's buffer, see Appendix) in 0.2% concentration.

The enzyme and water jet system was initially tested through removal of germlings from the surface of glass microscope slides. A drop of 0.0125% Yeast Extract Medium (St.Leger *et al.*, 1989) was placed in the centre of a sterilised glass microscope slide, inoculated with a drop of 1 x 10^6 conidia ml^{-1} suspension and incubated for periods of 24, 30 and 48 h at 27°C at 100% humidity. At these time intervals, the number of germinated conidia per 300 counted per slide was recorded, before the slides were subjected to one of 2 treatments. The first treatment was (i) a 20-second water jet only, and the second treatment (ii) was the enzyme solution (0.2% concentration) plus a 20sec water jet. For treatment (i) the glass slides, with germlings attached, were subjected to a 20-second water jet only. In treatment (ii) 1 ml buffered 0.2% laminarinase solution was applied to the centre of the microscope slides and incubated at 37°C (optimal temperature for the enzyme) for 24 h. After incubation the slides were then subjected to a 20-second water jet. The number of germinating spores still adhering to the surface of each slide was then determined after each treatment. Each treatment was replicated 5 times, and conidia were tested for their viability using the method described in part 2.1.1 of this Chapter. Treatments were compared using the non-parametric Kruskal Wallis test (full details of which can be found in the Appendices).

The laminarinase/water jet treatment was then tested on fungus treated locust hind wings 24 and 30 h after inoculation viz. when germination and appressoria formation had occurred (respectively) but prior to penetration of the hind wing cuticle by the fungus (hind wings were inoculated as previously described in part 2.2.6). Counts were performed on the hind wings before they were placed in 0.2% laminarinase solution for 24 hours and then subjected to a 20-second water jet, and after this procedure. Control treatments were subjected to the water jet treatment only and were not exposed to the laminarinase enzyme. The number of germinated conidia per field of view (at x 400 magnification under the light microscope) were counted for each treatment, and each treatment was replicated 5 times. Conidia were tested for viability prior to the experiment. The control, water jet only and laminarinase + water jet treatments were statistically tested with the Kruskal Wallis test (see Appendices).

2.4.2 Germination on wing previously attacked by fungus

The following experiment was designed to determine the extent of nutrient utilization by *M.anisopliae* var. *acridum* from the surface of *Schistocerca gregaria* hind wings.

Hind wings that had been previously attacked by the fungus for a period of 24 h were used for the experiment. These were prepared by spreading sterilised hind wings on 2% water agar plates (5 plates with 3 wings per plate) and inoculating them with 4 x 10^6 conidia ml^{-1} suspension in dH_2O. These wings were then incubated for 24 h at 100% humidity, before being immersed in a solution of laminarinase enzyme for a further 24 h, to remove germlings from the surface.

A control treatment was prepared using the same protocol as above, but where the wings were not inoculated with the fungus, but with the laminarinase solution only (5 plates, 3 wings per plate).

After the wings had been prepared and rinsed of the laminarinase solution with a water jet, they were again spread flat on freshly prepared 2% water agar plates, and inoculated with 4 x 10^6 conidia ml^{-1} suspension in dH_2O (5 plates for each treatment, 3 wings per plate). Germination counts were made, according to section 2.2.1 at 24 and 48 h post inoculation.

2.5.0 Analysis of epicuticular extracts

2.5.1 Analysis of epicuticular extracts by Gas Chromatography linked to Mass Spectrometry (GC-MS)

Pentane, chloroform and methanol extracts made from locust hind wings both before and after fungal attack, were analysed by Gas Chromatography linked to Mass Spectrometry (GC-MS). This technique enabled the separation and identification of the constituents of each extract.

Apparatus used to facilitate this analysis included a Hewlett Packard 5890A Gas Chromatograph, fitted with a 10 m CPSil 5 Chrompack column. This was linked to a Hewlett Packard 5970 Mass Selective Detector. Samples were injected, after being dried down and re-suspended in either ethyl acetate or hexane, onto the column manually. A 5µl SGE on-column syringe fitted with a fused silica needle was used and 2µl quantities were injected per run.

Various protocols for GC-MS analysis were developed for each type of extract, according to the predicted contents of each extract (the two employed most extensively are shown in the tables below). The time period for each GC-MS analysis varied between 20 and 50 min, depending on the method chosen. A gas chromatogram was produced for each sample and an Hewlett Packard software library was used to identify peaks produced on each chromatogram, using the mass spectra data.

Table 2.1: Method used for pentane and chloroform extracts and for quantification individual components (see 2.6.6)

°C/min	**Final Temperature (°C)**	**Final Time (min)**
25.0	150	0.00
10.0	245	5.00
5.0	290	15.00
Start Temperature	**Solvent delay**	**Total Run Time**
60°C	5 min	44.5 min

Table 2.2: Method used for analysis of derivatised methanol extract and TLC bands

°C/min	Final Temperature (°C)	Final Time (min)
10.0	120	0.00
5.0	220	0.00
10.0	290	5.00
Start Temperature	**Solvent delay**	**Total Run Time**
70°C	3 min	37 min

2.5.2 Methods for derivatisation of a sample for analysis by GC-MS

N, N-bis(trimethyl-silyl) trifluoroacetamide (BSTFA) a trimethylsilyl donor (Pierce Chemical Co. Ltd.) was used to derivatise fatty acids in the methanol extract. 5-10μg of the sample (dry) was combined with 500μl BSTFA and 1ml pyridine solvent in a 2ml vial. This mixture was shaken for 30 sec and then heated to 70°C for 15 min to facilitate silylation, before being analysed by GC-MS.

The second agent used for derivatisation of the methanol extract was Methyl-8® Concentrate reagent (N, N-Dimethylformamide dimethyl acetal, Pierce Chemical Co. Ltd.), recommended for methyl ester preparation from fatty acids and amino acids (Pierce Chemical Co. Ltd.). 50mg of sample was combined with 300μl Methyl-8® Concentrate in a 2ml vial. The vial was capped and heated for 60°C for 10-15 min, and the sample was then analysed by GC-MS.

2.5.3 Methods for quantification of individual epicuticular components

The procedure below was devised to demonstrate that an individual epicuticular component within an extract from a locust hind wing could be quantified both before and after fungal attack of the wing.

The pentane extract from the wing, which consisted of long chain hydrocarbons, was chosen as the extract, and octacosane (a 28-carbon *n*-alkane) was selected as the individual component to be quantified.

Docosane, a 22-carbon *n*-alkane, was selected as the internal standard, as this hydrocarbon did not occur in the pentane extract from the locust hind wing.

A 50µl ml^{-1} stock solution was prepared for each hydrocarbon in hexane solvent. This was diluted in hexane so that there were 3 different octacosane concentration solutions (as shown on table 2.3), while the amount of docosane was kept constant.

Table 2.3: Octacosane concentration solutions for quantification GC-MS analysis

Concentration octacosane (µg ml^{-1})	**Octacosane (µl)**	**Docosane (µl)**	**Hexane solvent (µl)**	**Total volume (µl)**
22.2	200	200	50	450
11.1	100	200	150	450
5.6	50	200	200	450

These solutions were then injected manually onto the column, as described in part 2.5.1 of this chapter, and a chromatogram produced for each.

Two peaks appeared on each chromatogram, representing docosane and octacosane respectively. Hewlett Packard software was then used to calculate the peak area of each peak on each chromatogram. These peak area values were used to create a peak area ratio (by dividing the octacosane value by the docosane value for each chromatogram) and these were used to plot a calibration curve for octacosane.

The octacosane calibration curve was plotted with the octacosane concentrations in $\mu g\ ml^{-1}$ on the 'x' axis and peak area ratio on the 'y' axis, on Minitab 11 for Windows. Regression analysis was then performed to derive the equation of the line of the graph. With this equation it was possible to estimate the quantity of octacosane present on 3 locust hind wings before and after fungal attack. This could be calculated after the peak area ratio for the octacosane present on those chromatograms produced for the pentane extracts pre and post fungal attack were determined and substituted into the equation of the line.

2.5.4 Glucose oxidase assay

A modification of the glucose oxidase assay (Trinder, 1969) was used to determine glucose in pentane, chloroform and methanol extracts. The extracts were dried down in their vials (HPLC Technology Ltd.) using Edwards vacuum freeze drying apparatus (Modulyo Ltd.) and rehydrated with 100µl sterilised dH_2O. Controls were prepared using the pentane, chloroform and methanol solvents (100µl of each) and a 0.01% glucose solution. These treatments were all introduced into wells of a Sero-

Wel® (Bibby Sterilin Ltd.) microtitre plate and to each well 150µl of Trinder reagent, containing 0.5mM 4-aminoantipyrine, 20mM p-hydroxybenzene sulphonate, 15,000 units/L glucose oxidase and 10,000 units/L horseradish peroxidase, was added. The reactions were allowed to occur at 27°C for 20 min and the concentration of glucose was determined by measuring the absorbance at 520nm in a Dynatech microtitre plate reader against a series of known glucose standards.

Control treatments of wings excised from the locust body, which had NOT been inoculated with fungus, but which had been treated under the same conditions as infected hind wings were prepared. These were extracted and assayed for glucose content to ensure that any change in glucose levels in the extract occurred as a result of the fungus rather than due to the effects of ageing of the wing.

2.5.5 Ninhydrin assay

The ninhydrin method (Samuels, *et al.*, 1993) was used to detect nitrogen-containing compounds in the pentane, chloroform and methanol extracts made from locust hind wings. The ninhydrin reagent was prepared immediately before use by mixing equal volumes of 4% (w/v) ninhydrin in 2-methoxyethanol with 0.2M citrate buffer (pH 5.0) containing 0.2% (w/w) $SnCl_2.H_2O$.

2ml of the ninhydrin reagent was added to a 100µl aliquot of each of the pentane, chloroform and methanol extracts (the extracts had been dried down and re-suspended in 100µl sterile dH_2O for this assay) and to the control preparations; 100µl pentane, chloroform and methanol solvent solutions and an 0.01% alanine (the

extract had been dried down and resuspended in 100µl sterile dH_2O for this assay). The mixture was then boiled for 20 min in capped test tubes in a water bath.

After cooling, 3 ml 50% (v/v) propan-2-ol was added. The solutions were then left to stand for 15 min prior to measuring the absorbance at 570 nm in a Cecil dual beam spectrophotometer.

Control treatments of wings that had been excised from the locust body, which had NOT been inoculated with fungus, but which had been treated under the same conditions otherwise as those which had, were extracted and assayed for protein content to ensure that the any change in protein levels in the extract occurred as a result of the fungus rather than due to the effects of ageing of the wing.

A calibration curve was generated for the assay using alanine as a standard. Results were expressed in terms of 'alanine equivalents'.

2.5.6. Amino acid analysis

Amino acid analysis of the methanol extract before fungal attack and after fungal attack (samples A and B respectively) was performed by Alta Bioscience, based at the University of Birmingham, UK. Analysis was performed on each sample before and after hydrolysis, in order to detect both free amino acids in solution, and any amino acids present as proteins and peptides in the samples.

Separation was based on the AltaBioscience method of ion exchange chromatography, followed by colourimetric detection by ninhydrin.

For separation and detection of free amino acids in solution, the sample was first buffered to a pH of 2, then injected onto the top of a column of strong cation exchange resin and heated in an oven to optimise separation. Amino acids were then eluted by a stepwise gradient of increasing pH, which separated them by their different pK's. Amino acids were then detected by colourimetric detection by ninhydrin.

For separation and detection of amino acids present in a sample as proteins and peptides, the samples were first hydrolysed. The samples were placed in evacuated sealed tubes and hydrolysed by constant boiling in HCl at 110°C for 24 h. Oxygen, which can destroy some of the amino acids, was removed from the samples by degassing (alternately freezing and thawing the samples under high vacuum). After hydrolysis, excess acid was removed by evaporation and the amino acid mixture was analysed as described for free amino acids in solution above.

2.5.7. TLC assay for monosaccharides

The separation and identification of monosaccharides from the methanol extract was achieved according to the method of Gal (1968). 5 µl of the methanol extract was spotted onto a silica gel 60 thin layer chromatography aluminium-backed plate (Merck), together with 5 µl of each three standards (glucose, galactose and *N*-acetyl glucosamine) at 2 cm intervals. The spots were placed at approximately 2cm from the bottom edge of the plate on a line parallel to it. The spots were then left to dry.

Meanwhile, a Shandon Ltd. Chromatank TLC tank was set up and lined with filter paper, and a solvent combination of *n*-propanol/water (7/1 v/v). When the spots on the TLC plate had dried the plate was placed inside the tank and the developing solvent was allowed to ascend to about 1 cm from the top of the TLC plate, which took approximately 4 h to complete.

After the developing solvent system had reached 1 cm from the top of the plate, the plate was removed from the tank and air-dried inside a fume cupboard.

A silver nitrate spray reagent was used to visualise monosaccharides. A solution of 3 g silver nitrate in 12ml dH_2O was added to 500 ml acetone. The plate was sprayed with this solution and dried, then sprayed with 0.5 M ethanolic (95%) sodium hydroxide solution (50ml 10M sodium hydroxide in 450ml absolute ethyl alcohol). Monosaccharides were detected as dark brown spots.

2.6.0 Germination on authentic compounds

2.6.1 Polar lipids

Authentic polar compounds were tested *in vitro* for their ability to support germination of *M.anisopliae* var. *acridum*. 1% (v/v) solutions of oleic acid (free fatty acid, $C_{18}H_{34}O_2$), oleic acid ethyl ester (free fatty acid ester, $C_{20}H_{38}O_2$), triolein (trisaccharide, $C_{57}H_{104}O_6$) and 1,3 diolein (disaccharide, $C_{39}H_{72}O_5$) were prepared in methanol solvent.

One drop of each solution was placed in the centre of a separate, sterilised glass microscope slide. 5 replicates were prepared for each solution, together with a control preparation of methanol solvent only. Slides were then placed on glass rods inside a petri dish lined with filter paper moistened with dH_2O and the solvent was allowed to evaporate off. The slides were then inoculated with a drop 1 x 10^6 conidia ml^{-1} suspension and incubated at 27°C and 100% humidity. Germination was recorded as the number of conidia germinated out of 300 counted per slide at 24 and 48 h post inoculation.

2.6.2 Non-polar lipids

Basal salts agar was formulated by adding basal salts (see Appendix for recipe) containing $NaNO_3$ (0.2% w/v) as an essential nitrogen source, to 2% agar (Oxoid Ltd.). A number of *n*-alkanes of various chain-lengths were provided as the sole carbon source.

Solid *n*-alkanes (see table 2.4) and the standard, *N*-acetyl glucosamine (readily available carbon source) were added at 1% w/v to 100 ml basal salts and 2% agar in a 250 ml conical flask and autoclaved. This solution was then poured into 5 petri dishes (Sterilin®) and allowed to set, to make basal salts agar plates.

Table 2.4: *n*-alkanes tested for ability to stimulate germination in *M.anisopliae* var. *acridum*

***n*-alkane**	**No. carbons**
Nonane	9
Tetradecane	14
Pentadecane	15
Hexadecane	16
Heptadecane	17
Docosane	22
Tetracosane	24
Hexacosane	26
Octacosane	28
Nonacosane	29
Triacontane	30
Hexatriacontane	36
Squalane	Branched alkane

Liquid hydrocarbons were filtered through Acrodisc® PF syringe filters (Gelman Sciences) and added to pre-autoclaved basal salts agar solution as it cooled, to make plates. 5 plates were made per 100 ml of solution.

The *n*-alkane basal salts agar plates were then inoculated with 3 x 10^6 conidia ml^{-1} suspension and incubatedfor 24 h at 27°C. Germination was assessed under the light microscope and the number of germinated conidia out of 300 conidia counted per plate was recorded for each treatment.

2.6.3 Glucose and amino acids

The following experiment was designed to confirm the estimated concentrations of glucose and peptides within the methanol extract taken from 3 locust hind wings (concentrations which were initially determined by assays detailed in parts 2.5.3 and 2.5.4 of this chapter).

Equivalent concentrations of authentic compounds were then tested for their ability to stimulate germination in *M.anisolpiae* var. *acridum* conidia, both alone and in combination. Nutrients tested included glucose, and the amino acids glycine and alanine (which represented the two most abundant amino acids found after amino acid analysis of the methanol extract). A 0.13mM solution of glucose, and 0.082µmoles solutions of alanine and of glycine were prepared, and various combinations of these were used, as listed below.

i) Glucose only

ii) Alanine only

iii) Glycine only

iv) Glycine and alanine

v) Glycine and glucose

vi) Alanine and glucose

vii) Glucose, alanine and glycine

A drop of each 'nutrient' solution was placed in the centre of separate sterilised glass microscope slides, which were in turn placed inside petri dish (Sterilin®) chambers lined with moist filter paper. Each preparation was then inoculated with a drop of 1 x 10^6 conidia ml^{-1} suspension in dH_2O. A control treatment was also prepared, where

a drop of conidia suspension only was placed on a sterilised microscope slide within a lined petri dish. 5 replicates were prepared for each treatment and these were all incubated at 27°C at 100% humidity. Germination counts were made at 30 and 48 h post inoculation, and the number germinated per 300 conidia counted per slide recorded.

2.6.4. Germination on pentane, chloroform and methanol extracts

5 µl of locust wing extract (the equivalent of 3 locust wings' extract, as 50 µl represented 50 wings' extract), either pentane, chloroform or methanol extract, was placed in the centre of a sterilised glass microscope slide. The solvent was allowed to evaporate leaving the extract on the surface of the slide. The extract was then inoculated with a drop of 1 x 10^6 conidia ml^{-1} suspension and the slide was placed on a bent glass rod inside a petri dish lined with moist sterilised filter paper. This preparation was incubated at 27°C for periods of 24 and 30 h post inoculation.

Control slides were also prepared, where 5 µl of each solvent only (pentane, chloroform and methanol) was placed on separate, sterilised slides within a petri dish, inoculated and incubated as described above. Measurement of germination were taken at 24 and 30 h post inoculation and number of conidia germinated per 300 counted per slide recorded for each treatment.

The same procedure was also followed to test those extracts taken from locust hind wings that had previously been attacked by fungus.

2.6.5. Germination on TLC bands separated from the methanol extract

The methanol extract was spotted onto silica gel 60 (Merck Ltd.) TLC plates using glass capillary tubes (HPLC Technology) in a line 2cm parallel to the base of the plate.

The plate was then allowed to develop in a hexane:diethyl ether:glacial acetic acid (90:10:2) solvent mixture in a Shandon Ltd. TLC Chromatank lined with filter paper for 2 h, or until the solvent front had reached within 1.5 cm of the top of the plate.

This procedure was repeated, but using different solvent system ratios. These were hexane:diethyl ether:acetic acid in 80:20:2 and 50:50:2 mixtures, which were used in order to achieve separation of different fractions from the methanol extract.

After the TLC plates had been developed in solvent systems, they were allowed to air dry inside a fume cupboard, and separated lipid fractions were visualised using iodine. Iodine crystals were placed within a separate chromatank and left to vaporise. The TLC plates were then introduced, and any lipid spots were coloured brown by the iodine vapour.

Lipid fractions on each plate developed in each different solvent system were labelled on the plates. These 'bands' were then scraped from the surface of the plate with a double-sided razor blade and placed in separate, appropriately labelled 3 ml vials (HPLC Technology). 1 ml of methanol solvent was then added to each vial to dissolve the fraction from the silica. The silica/lipid suspension in methanol was then taken up into a syringe and passed through a PTFE filter that attached to the

syringe (Acrodisc®, Gelman Sciences, Ltd.) to remove the silica, and into a new vial. This was repeated for each band from each TLC plate.

Each band was then examined for any ability to trigger germination in *M.anisopliae* var. *acridum*. To investigate this, a drop of each band extract was placed in the centre of separate, sterilised, glass microscope slides. 5 replicates were prepared for each band together with a control preparation of methanol solvent only.. These slides were then enclosed within petri dishes (Sterilin®) lined with sterilised, moist filter paper. Each slide was then inoculated with a drop of 1 x 10^6 conidia ml^{-1} in dH_2O, and incubated for 24 and 48 h, when germination counts were made. 300 conidia were counted per slide for each band, and the average number germinated recorded per band.

2.7 Hind wing structural studies

2.7.1 Cryo-scanning electron microscopy (Cryo-SEM)

The following protocol was followed for investigations into the surface structure of locust hind wings, using the Cryo-Scanning Electron Microscope system. A number of different treatments were applied to 10 day old (post ecdysis) hind wings, including:

i) sterilisation with propylene oxide (standard treatment)

ii) unsterilised hind wing,

iii) 24 hours of infection with *M.anisopliae* var. *acridum*

iv) treatment with laminarinase of a fungal-infected wing (to remove fungal growth)

v) uninfected hind wing treated with laminarinase

vi) extraction with pentane, chloroform and methanol

vii) axenic rearing conditions

viii) newly-moulted hind wings were also studied.

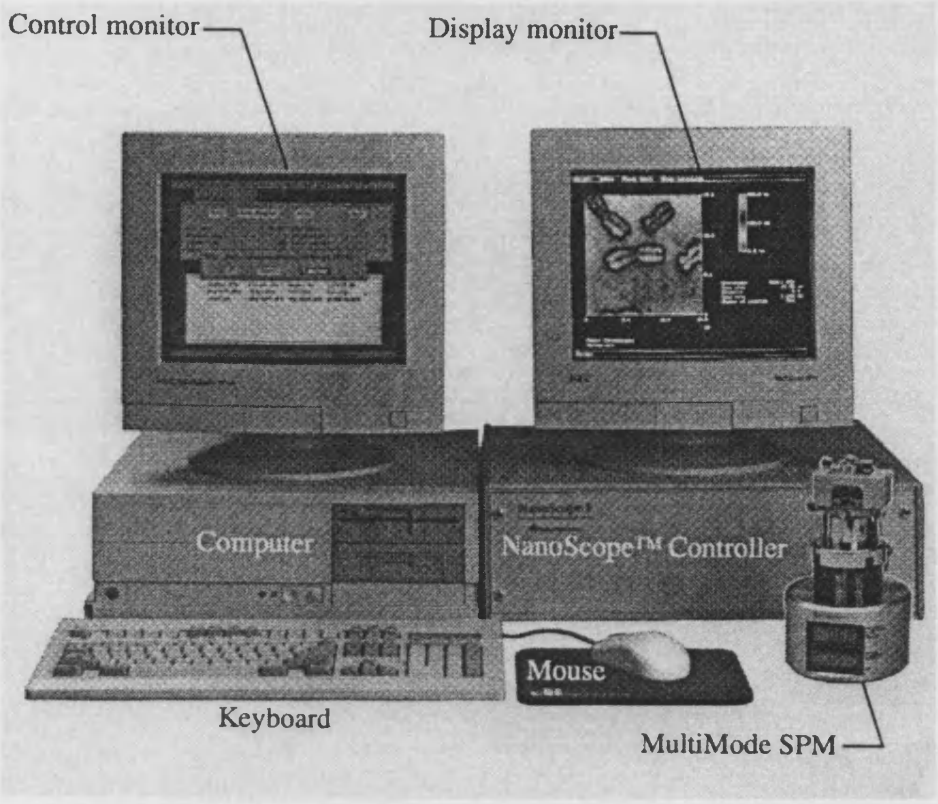
All of the treatments were investigated to ascertain if they caused any possible visible changes to the structure of the hind wing.

A small piece of about 1cm^2 was cut from the centre of the hind wing and mounted on a specimen holder. This was then placed in the slushing chamber of the Scanning Electron Microscope (SEM) and cooled rapidly with liquid nitrogen (0.5kg/cm^2) for 1-2 min. The specimen holder was then transferred to the cold stage of the SEM, which had previously been cooled with liquid nitrogen. Next, the specimen was examined at low kilovolts (KV) to determine whether detail had been obscured by frost. Any ice on the surface of the sample was sublimed off at –85-95°C. After sufficient ice had been removed, the specimen was transferred from the cold stage chamber to the Cryo- preparation stage. The specimen was then sputter coated with gold for a period of 2-3 min before being returned to the SEM cold stage and viewed under vacuum and 10-15 KV. A number of high and low power micrographs were obtained for each hind wing treatment.

2.7.2 Atomic Force Microscopy (AFM)

To achieve three-dimensional imaging and measurement of unstained and uncoated hind wing samples, the comparatively new technique of Atomic Force Microscopy (AFM) was employed. The apparatus is shown in Figure 2.4 below.

Figure 2.4: AFM apparatus (Digital Instruments Inc.).



Superior resolution (over SEM) can be attained by the AFM, which uses a cantilever, usually made from silicon or silicon nitride, on the end of which is a sharp tip used for imaging. When the tip is brought close to the sample surface, forces between the tip and sample cause the cantilever to bend. This motion is detected optically by the deflection of a laser beam, which is reflected off the back of the cantilever.

When the tip is scanned across the surface of the sample, the deflections of the cantilever can be recorded as an image, which represents the three-dimensional shape of the sample surface.

Hind wing samples were prepared by pressing a double-sided adhesive disc of approximately 1cm in diameter into the underside of the hind wing. A scalpel was then used to trim away the remaining wing piece. The disc was then turned over and the remaining adhesive coated side was placed on a further disc ready to be mounted on the sample stage of the MultiMode™ (Digital Instruments Inc.).

The specimen was then examined using Tapping Mode™ AFM, which allowed the measurement of topography by tapping the surface of the sample with an oscillating tip. Tapping Mode™ was chosen over other modes of the AFM as it eliminated shear forces that could have damaged the soft waxes on the epicuticular surface of the hind wing samples.

A number of different hind wing preparations were analysed using this method, including a wing sterilised with propylene oxide, a hind wing after it had been attacked by fungus (fungus removed with laminarinase enzyme and water jet

treatment), non-sterilised hind wings and axenic hind wings (those that had been excised from adult locusts which had been reared in axenic conditions). A series of three-dimensional images were produced for each type of hind wing, and their similarities and/or differences compared.

4 Discussion

The importance of germination and appressoria formation, the prerequisites for penetration and the first steps towards initiation of the fungal disease process in insects, has been long recognised and highlighted by many researchers (Charnley, 1989a, Gillespie and Moorhouse, 1989, Hajek and St. Leger, 1994, Clarkson and Charnley, 1996).

Work in section 3.1.0, Chapter 3, investigated the initial behaviour of the conidia of *M.anisopliae* var. *acridum*, isolate IMI 330189, when incorporated onto or into various nutrient and/or suspension media. Familiarization with the processes of germination and appressoria formation under various conditions *in vitro* allowed for future comparisons with the semi *in vivo* conditions of the locust hind wing bioassay system.

Low levels of germination were noted on the ¼ SDA medium (less than 2%) up to 12 h post inoculation with *M.anisopliae* var. *acridum,* (IMI 330189). These were very similar to those found by St. Leger and co-workers (1989) for *M.anisopliae* isolate ME1 on another nutrient-rich medium, 0.0125 % Yeast Extract Media (YEM). 100% germination was recorded at 14 h post inoculation by St. Leger *et al.*, (1989) and at 16 h with *M.anisopliae* var. *acridum.*

Appressoria formation in *M.anisopliae* var. *acridum* began at 18 h post inoculation and earlier (7 h post inoculation) for ME1 on YEM (St. Leger *et al.*, 1989). In both studies appressorial induction occurred before all conidia had germinated. Apart from the medium, a further difference between these two studies however, was that a polystyrene surface was provided in the ME1 study. No such hard surface was provided in this study. Terminal appressoria formation occurred in a mean of over 50% of *M.anisopliae* var. *acridum* conidia inoculated onto 2% water agar. In contrast, terminal appressoria formation in ME1 only occurred when germ tubes contacted a hard, hydrophobic surface (St. Leger *et al.*, 1989). There were no appressoria on 1.2 and 5% water agar, or when petri dishes containing 0.0125% YEM, locust cuticle peptone or alanine were swirled to prevent contact with the bottom of the dish.

Branched appressoria were observed on 9% water agar by St. Leger and co-workers (1989). In a later study St. Leger *et al.*, (1992), found considerable variability among isolates of *Metarhizium* spp. in ability to germinate and differentiate in various nutrient media, including YEM, glucose, Sabouraud's Dextrose Broth and YEM supplemented with chitin. Most isolates from scarabaeids germinated readily only in the presence of a crude chitin/protein product, whilst isolates from other Coleopterans and Hemipterans were much more adaptable and produced appressoria in YEM and glucose media respectively (St. Leger *et al.*, 1992). No Orthopteran-derived *M.anisopliae* isolates were examined.

Tween 80 and dH_2O, used as conidia harvesting, storage and delivery media, were compared for their ability to induce germination *in vitro* in *M.anisopliae* var.

acridum (IMI 330189). Tween 80 (polysorbate 80) is a hydrophilic surfactant (surface active agent) used widely in the harvesting and application of conidia from *M.anisopliae* spp. (Joshi *et al.,* 1992), facilitating a uniform suspension of usually hydrophobic conidia.

Significant germination was observed in 0.04% Tween 80 solution (a mean of ~31% germination 16 h post inoculation). Though this was less than in ¼ SDA medium (a mean of 100% germination, 16 h post inoculation), it was significantly greater than in dH_2O at 16 h post inoculation (a mean of ~4%). Since a low basal germination rate was necessary to assess the effects of desert locust cuticular components on the germination and appressoria formation of *M.anisopliae* var. *acridum,* the use of Tween 80 solution for harvesting, storage and conidia delivery medium was discontinued for the present work. Sonication of dH_2O reduced clumping in the hydrophobic conidia. Boucias and Pendland (1991) reported that harsh, prolonged sonication of *M.anisopliae* conidia could disrupt an outer conidial layer comprising interwoven fascicles of rodlets which are thought to aid adhesion. No detrimental effects of sonication were observed in the present work.

Ondina El oil is the standard formulation in the work of LUBILOSA because it promotes germination at lower humidities (Bateman *et al.*, 1993). Oil formualtions also provide conidial protection from UV radiation and high temperatures during storage and in the field (Lomer *et al.*, 1993, Moore *et al.*, 1993, Stathers, *et al.*, 1993, McClatchie *et al.*, 1994, Hooper *et al.*, 1995, Morley-Davies *et al.*, 1995 and Moore *et al*, 1996). Interestingly, conidia of *M.anisopliae* var. *acridum* germinated faster and in higher numbers when applied to water agar in Ondina El oil, than when

presented in dH_2O, at 24, 30 and 48 h post inoculation. This suggests that some component(s) of Ondina El oil support(s) low level germination. In the light of this, Ondina El oil was not used in the harvesting, storage or delivery *in vitro* or *in vivo* of *M.anisopliae* var. *acridum* conidia for the remainder of this project.

Washed spores did not germinate significantly in distilled water (dH_2O). Dillon and Charnley (1988) observed similar results and also found that soaking of *M.anisopliae* ME1 conidia in distilled water for 24 h accelerated germination once a suitable nutrient source was provided. Hassan *et al.*, (1989) and Milner *et al.*, (1991) found that pre-soaking increased pathogenicity of entomopathogensic fungi to insects.

Germination on sterilised (standard preparation), non-sterilised and axenic-reared excised desert locust hind wings was found to be faster and more extensive than on ¼ SDA media. It seems counter intuitive that conidia should germinate faster on the apparently unpromising medium of a locust wing than on a rich, readily available nutrient source. Ability to germinate well on wings may have implications for locust control.

51% of an oil formulation of *M.anisopliae* var. *acridum* conidia, sprayed by ULV apparatus, landed on the wings of desert locusts in flight (James, 1995). It is also known that penetrant hyphae grow and spread within the bilayer of cuticle (James, 1995) that forms the two membranes of the wing in between veins (Uvarov, 1966, Schabel, 1978). Therefore, potentially the fungus could invade the body of the insect from the wing via the system of fine veins (hollow tubes of sclerotised cuticle) which carry blood, and connect nerves and trachae to and from the insect haemocoel.

No significant differences were observed between germination on the different hind wing treatments tested. The fact that germination was similar on the sterilised and non-sterilised hind wing preparations suggests that propylene oxide, used as a sterilising agent throughout the present work, had no adverse effect on the germination of *M.anisopliae* var. *acridum*, confirming the work of James (1995).

James (1995) found that three isolates of *M.anisopliae* var. *acridum* (= *M.flavoviride*), including IMI 330189, germinated well on excised desert locust hind wings. However, abnormal germ tube growth followed. No aberrant growth was noted in any of the hind wings treatments detailed here. Terminal appressoria formation initiated at 12-18 h post inoculation on sterilised and non-sterilised hind wing resulted in ca. 47% appressoria at 30 h post inoculation. James (1995) found late formation of appressoria in 2 out of 3 *M.anisopliae* var. *acridum* isolates tested (including IMI 330189), and attributed this and abnormal germ tube growth to *post mortem* changes in wing chemistry from the locust and/or the chemical effects of propylene oxide. However, in the present work, there were negligible changes in concentrations of amino acids, protein and glucose in the 24 h after removal of the hind wing from the insect body. Hind wings were also used in experiments directly after their removal from live insects to avoid *post mortem* changes.

Appressoria formation was delayed on hind wings taken from locusts reared under axenic conditions. Appressoria did not occur until 24 h post inoculation and the numbers that occurred (ca. 23% at 24 h and ca. 36% at 30 h) were significantly lower than on sterilised and non-sterilised hind wings. This suggests that the surface

microflora of the wing may alter the chemistry or topography of the cuticle in such a way as to promote differentiation of appressoria, perhaps by providing optimal nutrients or cues (signals). However, there are no precedents for specific regulation of differentiation among isolates of *Metarhizium* spp.

The specificity of entomopathogenic fungi was defined by Fargues (1984) as the expression of reciprocal adaptations and affinities between a pathogenic organism and the entirety of its host species. As discussed in Chapter 1, entomopathogenic fungi have widely differing host ranges (Bridge *et al.*, 1993), with some isolates of *M.anisopliae* (*e.g.* ME1) infecting many different insects from various Orders, and others being highly specific, (*e.g. M.anisopliae* var. *acridum* IMI 330189, infecting only locusts and grasshopper hosts, Order: Orthoptera). The basis of specificity is poorly understood. Germination of *M.anisopliae* var. *acridum* IMI 330 189 occurred on sterilised excised hind wings from non-host *Calliphora vomitoria* (blowfly) and *Tenebrio molitor* (flour beetle), however, significantly more slowly and less extensively than on sterilised (standard preparation) wings of the desert locust. Germination on *C.vomitoria* wings was just ca. 6 % of conidia at 18 h post inoculation and 13% at 24 h post inoculation. Germination on desert locust hind wings, however was ca. 56% of conidia at 12 h post inoculation. Appressoria formation was better than germination on non-host wings. Approximately ca. 20% of conidia on *C.vomitoria* wings differentiated into appressoria, while ca. 89% of conidia formed appressoria on *T.molitor* hind wings (24 h post inoculation). By comparison, ~46% of conidia formed appressoria at 30 h post inoculation on desert locust hind wings. The reduced germination performance of *M.anisopliae* var. *acridum* on non-host wings, though significant, may not by itself be enough to account for specificity. However, it could be an important contributary

factor. Failure of the fungus to infect an insect species may result from a block at penetration, an inability to overcome host blood-borne defences *etc.* The increase in appressoria formation observed on *T.molitor* could be exploited.

Ball *et al.*, (1994) infected *Apis mellifera* (the honeybee, Order Hymenoptera), with *M.anisopliae* var. *acridum* IMI330189 although at a low level (11%) compared with *S.gregaria* (95%) at the same dose (Bateman *et al.*, 1993). Honeybees are easily stressed under laboratory conditions which predisposes them to disease. Apart from this study, available evidence is consistent with IMI330189 being specific for acridids (Moore, per. comm.). The basis of this interaction remains to be discovered.

Conidia germinated equally well on pentane and chloroform treated desert locust hind wings and on sterilised untreated wings. Thus these non-polar solvents did not remove nutrients/signalling molecules necessary for germination of *M.anisopliae* var. *acridum*. Germination did not occur at 12 h and was minimal at 18 and 24 h post inoculation on methanol treated hind wings, however, suggesting that nutrient or signalling cues may have been removed by the methanol solvent treatment.

Although pentane and chloroform treatments had no effect on germination, they promoted appressoria formation. Significantly more appressoria formed on those hind wings treated with pentane (24% of conidia) and chloroform (32% of conidia) and methanol (36% conidia) solvents at 18 h post inoculation than on the standard sterilised hind wings (13% of conidia) at 18 h post inoculation. This could be due to:
(a) the partial removal of non-polar compound(s) inhibitory to appressoria formation

(b) the removal of non-polar compound(s) that were masking nutrients or signalling molecules.

The data point towards the presence of a non-polar differentiation inhibitor, on the surface of desert locust hind wings.

Methanol treatments resulted in higher levels of appressoria formation at 18 h and lower levels at 24 h post inoculation, suggesting the presence of nutrient compounds that promote appressoria formation before 24 h post inoculation.

The results for germination on extracted hind wings were supported by studies on the extracts themselves. High levels of germination (75%) occurred on the methanol extract 24 h post inoculation, whilst minimal germination (16 %) occurred on hind wings extracted with methanol. From this we may conclude that methanol removed nutrients or other stimulants. Minimal germination occurred on pentane and chloroform extracts, whilst high levels of germination were recorded before and after extraction. Thus the effect of the non-polar solvents appears to be neutral.

Polar cuticular lipids from other insect classes have also been shown to stimulate germ tube growth (Boucias and Pendland, 1984, Boucias and Latgé, 1988 and Latgé *et al.*, 1987), though Lecuona *et al.*, (1997) found that the methanol extract from the whole body cuticle of *M.melolontha* inhibited both virulent and avirulent strains of the fungus.

Various workers have reported that non-polar solvent extracts of insect whole body cuticle may either inhibit germination (Koidsumi, 1957, Smith and Grula, 1982) or

result in germ tubes that are not capable of penetrating the host cuticle (Kerwin, 1984, Latgé *et al.*, 1987). Other work has also shown stimulation of germination by cuticle extracts. Polar lipid classes were found to stimulate germ tube growth (Boucias and Pendland, 1984, Boucias and Latgé, 1988 and Latgé *et al.*, 1987) whilst St. Leger *et al.*, 1988, and Napolitano and Juarez (1997) showed growth of *M.anisopliae* (ME1) on non-polar hydrocarbons, *n*-alkanes, as a sole carbon source.

M.anisopliae var. *acridum* IMI 330189 will also germinate (growth was not determined) on hydrocarbons. *n*-alkanes (particularly long-chain forms) incorporated into a basal salts mixture, induced high levels of germination 24 h after inoculation. GC-MS analysis revealed that longer chain hydrocarbons are the main constituents of the pentane and chloroform extracts from locust hind wings. Indeed they are the most common and abundant class of cuticular lipid in all members of the family Acrididae (shown by Jackson, 1982, Lockey and Oraha, 1990).

GC-MS is an effective analytical tool and provided the identities of many compounds present in the extracts. However, it is a destructive method that does not allow collection of individual components for testing. Hence, during this project, authentic lipids were used to determine the ability of individual lipids to support germination.

The ability of single, authentic *n*-alkanes to support germination must be contrasted with the low levels of germination on pentane and chloroform extracts. It is not clear

why this should be the case. Interestingly, Napolitano and Juarez (1997) found that mixtures of insect-derived hydrocarbons were more efficient as metabolic fuel for *M.anisopliae* (work did not include IMI 330189) and *B.bassiana* isolates than similar authentic hydrocarbon compounds. The opposite was found to be true in the present work using the more specific *M.anisopliae* var. *acridum* isolate (IMI 330189).

The crude methanol extract from locust hind wings stimulated high levels of germination compared to the pentane and chloroform extracts. The extract contained polar non-lipid compounds viz. glucose and amino acids, as well as fatty acids, esters and a very small amount of non-polar *n*-alkanes and methylalkanes.

The presence of *n*-alkanes and methylalkanes in the methanol extract, although in very small amounts, was not expected, particularly after previous treatment of the wings with pentane and chloroform. This may therefore suggest that either the extraction process used in this work was not rigorous enough, or that there is a subtle layering of lipids on the wing surface such that the underlying hydrocarbons are protected from extraction until the wings are treated with a more polar solvent. The latter seems the most likely, as, at 1h refluxing with each solvent (pentane, chloroform and methanol) the extraction procedure was sufficiently rigorous.

Previous studies have shown the presence of di- and triacylglycerol (Oraha and Lockey, 1990). Therefore a wider range of authentic polar lipids than those found in the crude methanol extract were tested for their ability to support germination in *in vitro* studies. The authentic diacylglycerol, triacylglycerol and fatty acid stimulated

germination. A fatty acid ester, however, produced a low response. However, in all cases, the germination observed occurred at a much slower rate on authentic polar compounds than on crude wing extract containing a mixture of polar compounds.

Kerwin (1984) suggested that the specificity of *Erynia variabilis* to adult dipteran hosts could be due to the combination of free fatty acids present on the cuticle of the hosts. In particular, on *Fannia canicularis* (the lesser housefly), the balance of stimulatory and inhibitory fatty acids was the key to successful germination. Kerwin found that high concentrations of linoleic acid were deleterious to germination. This was not the case on locust cuticle, since the methanol extract supported good germination of *M.anisopliae* var. *acridum*, yet contained linoleic acid (9.12-octadecadienoic acid). Barnes and Moore (1997) also found that linoleic acid gave supported 88.9% germination of *M.anisopliae* var. *acridum*. Furthermore, band 1 from TLC separation of a methanol extract appeared to consist solely of linoleic acid (9,12,-octadecadienoic acid), and supported high levels of germination in *M.anisopliae* var. *acridum*.

A further unsaturated fatty acid, oleic acid (9-octadecenoic acid), was highlighted by Kerwin as stimulatory to the germination of *E.variabilis*. No free oleic acid was detected in the desert locust wing extract in the present work, but its ethyl ester (Ethyl-2-hydroxy-9-octadecenoate) was present. Good germination of *M.anisopliae* var. *acridum* occurred on authentic oleic acid. Oraha and Lockey (1990) recorded 18-carbon fatty acids among the polar lipids of the cuticle of *S.gregaria* and *Locusta migratoria migratoroides* but did not state which 18-carbon fatty acids were present. Smith and Grula (1982) showed that nonanoic acid was inhibitory to the germination

of *B.bassiana* on corn earworm. This fatty acid was absent from the methanol extract of the locust cuticle in the present work. However, pentadecanoic, tetradecanoic (myristic), hexadecanoic (palmitic) and heptadecanoic acids recorded in the methanol extract may contribute to the growth of the fungus, as germination occurred on the authentic compounds of this type (Moore, pers. comm.).

Germ tubes of *M.anisopliae* adhere to the cuticular surface of their hosts by an amorphous mucus which is secreted by the hyphal tip (Zacharuk, 1970, St. Leger 1988a). A similar adhesive produced by *N.rileyi* comprised β1,3 glucans (Boucias pers. comm. in Charnley, 1989a, Boucias and Pendland, 1991). In this work it was found that laminarinase, an enzyme that hydrolyses β 1,3 glucans, and a water jet successfully removed germlings from glass microscope slides, and desert locust hind wings, at 30 h post inoculation. It would therefore appear that β 1,3 glucans must constitute a substantial component of the extracellular mucilage secreted by germ tubes and appressoria of *M.anisopliae* var. *acridum*; evident in cryo-SEM germlings (section 3.5.1, Figure 3.40).

Pentane, chloroform and methanol extracts were made before and after 24 hours of fungal growth (germination and appressoria formation). The pentane extract post-fungus revealed that some *n*-alkanes (pentacosane and tetracontane) and cholesterol present in the original pre-fungus extract, were completely absent, indicating use by the fungus, whilst all other *n*-alkanes had been dramatically reduced in quantity. In the chloroform extract, no peaks were identified after the 24 hour treatment with the fungus, suggesting that the fungus utilised all of the fatty acids and alkanes present.

One compound was detected and identified as N-[2-methyl propyl] 9, octadecenamide, in the post fungus methanol extract. This compound was not present pre-fungus, and thus it *may* derive from the fungus. No compounds identified in the methanol extract pre-fungus were present post-fungus, again suggesting complete usage by the fungus during the first 24 h post-inoculation.

Fungal utilisation of cuticular lipid was suggested by the appearance of the wing surface observed under cryo-SEM and Atomic Force Microscope, after the cuticle had been briefly (~ 24 h) exposed to germinating fungus. Removal of the fungus by laminarinase treatment allowed the first high magnification micrographs of an insect cuticular surface post-fungal attack (Plate Four, Chapter 3). Low magnification revealed 'clearing zones' in post-fungus hind wing surfaces, where areas of cuticular lipids appeared to have been removed. Higher resolution and magnification images produced by AFM suggested not complete removal but selectivity on the part of the fungus (Plate Eleven, Chapter 3). The AFM study revealed a highly corrugated surface after removal of fungus, rather than the initially smooth surface, consistent with selective utilisation. Recognition of a host by conidia of an entomopathogenic fungus can be due to the topographical signals present on the host surface, and can affect germination and appressoria formation (Hajek and St. Leger, 1994).

An initial attempt was made to quantify hydrocarbon use from the concentration (μg ml^{-1}) of *n*-octacosane, in the pentane extract before and after inoculation with *M.anisopliae* var. *acridum.* The results indicated 83.7% use of available octacosane during the 24 hours following conidial application. Similar work by Lecuona *et al.,* (1991) showed that 32-51% of total cuticular hydrocarbons of *M.melolontha* and 77-

86% of those of *O.nubilalis* had been affected by *B.bassiana* and *B.brogniartii* respectively. However, their study was conducted 96h post-inoculation, during which time considerable fungal growth will have occurred on and in the cuticle. Since their method did not include removal of fungus prior to lipid extraction, both insect and fungal hydrocarbons were determined. A clear distinction between the two was not possible; a fact also recognised by the authors. The spray application of conidia to both surfaces of the wing in the present work ensured even coverage, adding credence to the attempts at quantification of hydrocarbon use. Lecuona *et al.*, (1991) used a dip method of inoculation and did not determine its efficiency. The present work also allowed for distinction between individual hydrocarbon components, whilst previous work (Lecuona *et al.*, 1991) investigated hydrocarbons as a group.

The reduction of *n*-octacosane levels on the surface of the locust hind wing could have been brought about in part by deterioration of the detached hind wing. Later experiments, however, (discussed below) suggested no *post mortem* changes in at least glucose and protein 24 h after wings had been removed from the locust.

Glucose was only detected in the methanol extract due to its polar nature. Negligible amounts of glucose were found in the pentane and chloroform extracts. The level of glucose dropped by 80.6% after fungal inoculation. Monosaccharide and disaccharide residues were also detected in TLC analysis of the methanol extract (section 3.3.8, Chapter 3). Bands corresponding to mono- and disaccharide were greatly reduced in number and visibility in the post-fungus extract. Thus low MW

carbohydrates appear to make a contribution to pre-penetration fungal growth ≤ 24 h post inoculation.

Soluble compounds containing amino groups were detected in significant amounts using the ninhydrin assay, in the methanol extract. 67.4% less was found in the post-fungus extract indicating use during germination and appressorium formation. Peptides and amino acids probably made up most of the ninhydrin-positive material. Further, detailed analysis of the methanol extract confirmed the presence of both, before and after fungal attack. Large reductions in each amino acid occurred in the post-fungus extract.

It is not clear where the carbohydrates, amino acids and peptides found in the wing cuticle extracts have come from. They have not previously been recorded as constituents of the epicuticle or cement of the locust wing. However, such components were also found by Woods and Grula (1981) in a polar extract from the cuticle of the Lepidopteran *Heliothis zea*.

The amino acid composition of the methanol extract pre-treatment was similar to that determined by St. Leger *et al.* (1986a) following proteolytic hydrolysis of locust whole cuticle. Therefore these compounds may come from the body of the cuticle, perhaps *via* pore canals. Alternatively, harsh solvent treatment could remove peptides from the procuticle, giving a contaminated epicuticular extract. The latter does not appear to be the case. Fungal growth only occurred on the surface of the cuticle 24 h post-inoculation (present work and James, 1995). Therefore, the reduced concentration of amino acids and peptides in the post-fungus extract is consistent

with a superficial location for these compounds. Sugars on the surface of the insect cuticle may result from contact with plant, substrate, host fecal material or microbes on the hind wing surface (Boucias and Pendland, 1991).

Good germination occurred *in vitro* on glucose. Germination was poor on amino acids alanine and glycine (most abundant amino acids found by amino acid analysis to be present in the methanol extract) alone and, surprisingly, on amino acid/glucose combinations. This poor germination may have been due to the lack of a complex mix of nutrients, and may suggest that the carbon source is a more effective trigger for germination than the nitrogen source. Germination and germ tube extension of *M.anisopliae var. anisopliae* ME1 (St. Leger *et al.*, 1986a) and *Beauveria bassiana* (Woods and Grula, 1984) were supported by glucose and/or amino acids (particularly alanine and glycine). It is interesting that *M.anisopliae* var. *acridum* 330189, a more specific pathogen than either of the above, should have such a fundamental difference in nutritional requirements. It is not known why this should be so.

4.1.0. CONCLUSION

Conclusions can be drawn from the present work regarding utilisation by *M.anisopliae* var. *acridum* of cuticular components of host *S.gregaria*, the pre-penetration behaviour of its conidia, and its unique specificity for acridids during the first 24 hours of inoculation.

Surface components of the desert locust epicuticle were examined in detail and included a broader spectrum of components (*e.g.* glucose, peptides and amino acids, polar and non-polar lipids) than solely the lipid groups focussed on by previous workers (Jackson, 1982, Genin *et al.*, 1986, Oraha and Lockey, 1990, Lockey and Oraha, 1990, Buckner *et al.*, 1996). *M.anisopliae* var. *acridum* (IMI 330189) can germinate on authentic non-polar alkanes as a sole carbon source, preferring longer chain alkanes. *M.anisopliae* var. *acridum* is similar to *M.anisopliae* isolate ME1 (St. Leger *et al.*, 1988a) in this way. Only low levels of germination were observed however, in *M.anisopliae* var. *acridum* on crude, non-polar extracts, containing long-chain *n*-alkanes, taken from *S.gregaria,* where non-polar compounds were presented as a mixture. Napolitano and Juarez (1997) found good germination of entomopathogenic fungi (including a less specific *M.anisopliae* isolate) on a crude non-polar extract of *Triatoma infestans*.

Although *M.anisopliae* var. *acridum* IMI330189 conidia did not germinate well on non-polar crude hind wing extract, laminarinase treatment and GC-MS analysis showed that a large proportion of non-polar components were removed by the fungus during the first 24 h of inoculation. For example there was 83.7% less octacosane in

a non-polar hind wing extract after inoculation with *M.anisopliae* var. *acridum* for 24 h.

Meanwhile, excellent germination was observed on the polar crude extract from *S.gregaria* hind wings which GC-MS analysis and assays showed to contain fatty acids, fatty acid esters, glucose, amino acids and peptides, and on authentic glucose and fatty acids. Corresponding poor germination was observed on a locust hind wing from which the extract had been taken.

Glucose or other simple polar compounds could possibly be required to stimulate germination before the fungus can make use of the complex mixture of non-polar lipids.

Linoleic acid, a fatty acid, was identified through TLC and then GC-MS analysis as the epicuticular component which stimulated the greatest level of germination of *M.anisopliae* var. *acridum in vivo.* TLC was used to separate linoleic acid from the methanol extract. The separated components were then inoculated with *M.anisopliae var. acridum.* One suggestion arising from the present work would be to add this fatty acid to oil formulations used in the field. Barnes and Moore (1997) added a variety of authentic fatty acids to the oil formulation and tested for their ability to induce germination in *M.anisopliae* var. *acridum.* They found that certain fatty acids of chain length C10 and below produced significant inhibition of germination, whilst longer chain fatty acids produced no inhibition of germination. The greatest germination of *M.anisopliae* var. *acridum* conidia occurred, in fact, when linoleic acid was added to oil formulation (88.9%). The work of Barnes and Moore (1997) is

supported by the work here, in that it shows that a long chain fatty acid (presented to the fungus alone rather than in oil formulation) can induce high levels of germination in *M.anisopliae* var. *acridum* conidia. However, this work differs in that it has singled out one fatty acid, as opposed to the work of Barnes and Moore (1997) that investigated a larger variety of long chain fatty acids. These fatty acids were, in fact, authentic compounds, unlike the fatty acids extracted from the locust cuticle itself used in the present work. Further work on the relationship between the germination behaviour of *M.anisopliae* var. *acridum* conidia and linoleic acid may be very worthwhile.

The present work shows poor germination of *M.anisopliae* var. *acridum* IMI330189 on authentic amino acids (alanine and glycine) and on an amino acid/glucose mixture, but good germination on authentic glucose solution, (suggesting a carbon source to be more effective at stimulating germination than a nitrogen source). The opposite was found by other workers for the less specific pathogens *M.anisopliae* var. *anisopliae* ME1 (St. Leger *et al.*, 1986a) and *B.bassiana*. The reduction in amino acids, as well as mono- and di-saccharides in whole extracts from desert locust hind wings during 24 h of inoculation with *M.anisopliae* var. *acridum,* however, suggests *in vivo* utilisation. This reduction was unrelated to *post mortem* changes in the wing.

M.anisopliae var. *acridum* IMI330189, a highly specific pathogen, appears to have fundamental differences in nutritional requirements highlighted by the studies on surface component utilisation. This may contribute to its specificity to acridids.

4.2.0 Future Work

The fungus removal system developed in this project provides more accurate estimations of lipid utilisation *in vivo* than previous work (Lecuona *et al.*, 1991). It would be useful to study composition of extracts at shorter intervals (*e.g.* 5, 10, 15 and 20 h) post-inoculation, to obtain a more accurate picture of nutrient utilisation during pre-penetration growth, *i.e.* pre-germination, germination, post-germination, and appressoria formation. Quantitative studies could be supported with AFM and Cryo-SEM images to see changes in micro-architecture of the cuticle. Important nutrients to each pre-penetration stage could then be incorporated into a mycoinsecticide delivery medium immediately prior to application to the pest.

To complement the present work on external nutrients used by *M.ansiopliae* var. *acridum*, lipids contained within the conidia could be studied. Endogenous lipids could be extracted from conidia using the same solvent systems and GC-MS analysis employed in this project, in order to determine the nutrients present and their deployment during germination. Although external nutrients are required to initiate germination of *M.anisopliae* var. *acridum* conidia (the conidia are "nutrient insufficient", Boucias and Pendland, 1991) the process may be maintained, in the short term, by endogenous nutrients.

A distinction needs to be made between insect cuticular components used as nutrients and those used as cues by *M.ansiopliae* var. *acridum*. A non-metabolisable trigger of germination, such as methyl glucoside would be required for such experiments. Such investigations may provide us with answers regarding the specificity of *M.anisopliae* var. *acridum*.

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3 Results

3.1.0 The fungus and what it can do

3.1.1 Germination and appressoria formation *in vitro* on a rich, complete medium

Figure 3.1 shows the pattern of germination of *M.anisopliae* var. *acridum* on ¼ strength Sabouraud's Dextrose Agar (¼ SDA), a rich, complete medium.

There was a low level of germination 10-12 h post inoculation. Significant germination occurred at 12-14 h inoculation. By 16 h post inoculation almost total germination of conidia had taken place (a mean of 262.76 ± 21.1 out of 300).
Significant appressoria formation had taken place by 18 h (Fig. 3.2). In fact the first appressoria formed before the last conidia had germinated. Appressoria were terminal as opposed to branched in formation.

At 30 h post inoculation appressoria had reached a mean of 143.4 ± 7.7 per 300 conidia – almost half of all conidia counted had appressoria on their germ tubes.

The study was continued until 48 h post inoculation, but no further increase in the number of appressoria was recorded. Thus on ¼ SDA media in 2% agar, only one half of the conidia give rise to appressoria.

Figure 3.1: Germination of *M.anisopliae* var. *acridum* on ¼ SDA (mean ± sd, n = 25)

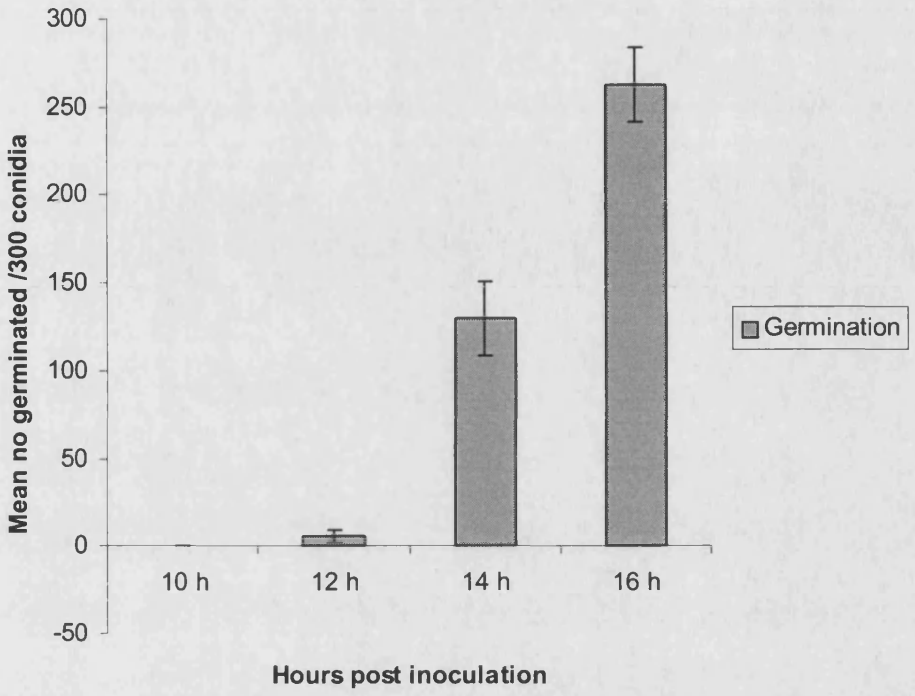
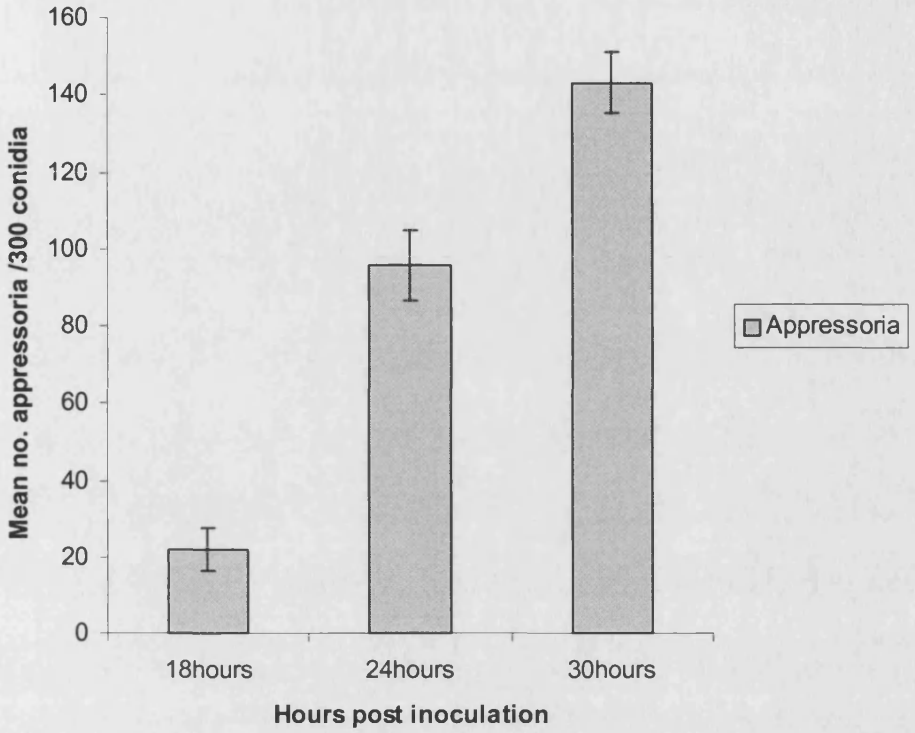


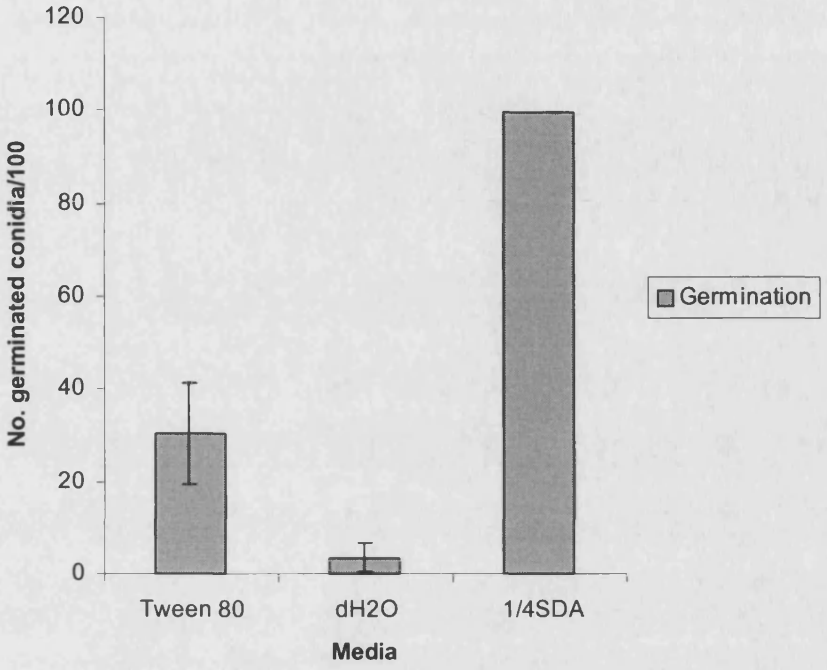
Figure 3.2: Appressoria formation of *M.anisopliae* var. *acridum* on ¼ SDA (mean ± sd, n=25)



3.1.2 Germination in 0.04% Tween 80 *vs.* dH_2O

Germination in 0.04% Tween 80 compared to dH_2O, 16 h post inoculation, is shown in Figure 3.3. Germination is significantly greater in O.04% Tween 80 (30.52 ± 10.94 per 100 conidia) than in dH_2O (3.56 ± 3.34 per 100 conidia). The germination level achieved in Tween 80 is significantly less than in nutrient rich medium (¼ SDA.

Figure 3.3: Germination of *M.anisopliae* var. *acridum* in 0.04% Tween 80 *vs* dH_2O and a nutrient rich medium (mean ± sd, n=96 replicates see section 2.2.3 Materials and Methods). Time = 16 h post inoculation.



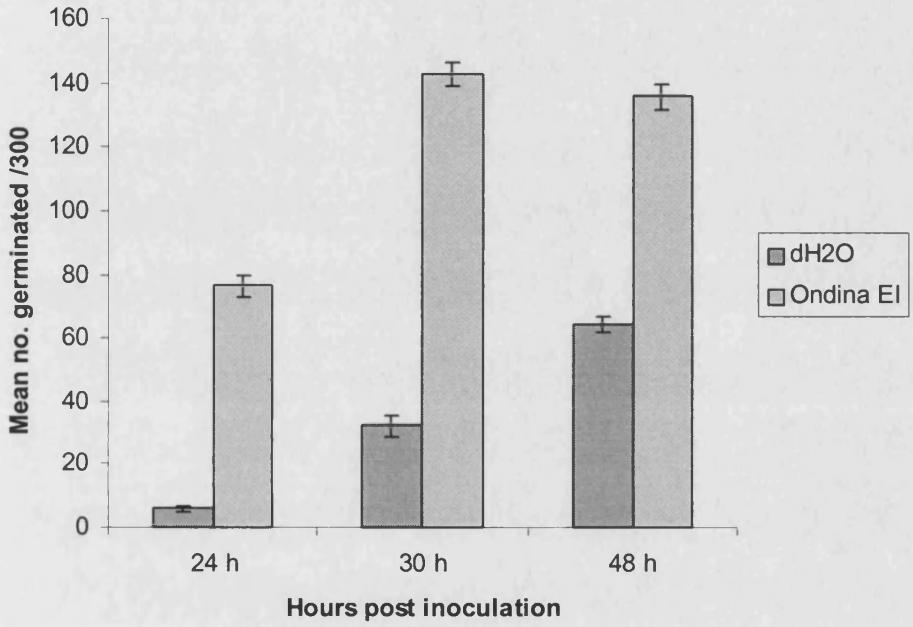
3.1.3 Germination in Ondina El oil

Ondina El oil is the primary delivery and storage medium for *M.anisopliae* var. *acridum* in field trials and for the Green Muscle™ commercial product developed as part of the LUBILOSA project. The ability of the oil to support germination was investigated.

Conidia were applied in oil or sterile dH_2O to water agar plates. Germination was determined at 24, 30 and 48 h post inoculation. Figure 3.4 shows the results of the study.

Conidia applied in oil germinated significantly more quickly and to a greater extent than those applied in dH_2O. Peak germination in oil was ca. 47% at 30 h, which is considerably less than that achieved on ¼ SDA, viz. 90% at 16 h

Figure 3.4: Germination of *M.anisopliae* var. *acridum* on dH_2O *vs.* Ondina El (mean ± sd, n = 25)



3.1.4 Germination and appressoria formation on *S.gregaria* hind wings

Germination

Figures 3.5, 3.6 and 3.7 show the pattern of germination on sterilised, non-sterilised and axenic locust wings respectively. The pattern was essentially similar in all three treatments. No, or minimal germination occurred 6-8 h post inoculation. Substantial germination occurred 10-12 h. In each case ca. 90% germination had occurred by 12 h. There were no significant differences between treatments ($p<0.01$, Kruskal Wallis

test, see Appendices). However, germination was significantly faster and more extensive on the excised locust wings than on ¼ SDA 12 h post inoculation (Mann-Whitney U test, $p<0.05$, Appendix).

Figure 3.5: Pattern of germination of *M.anisopliae* var. *acridum* on sterilised *S.gregaria* hind wings (mean ± sd, n= 25)

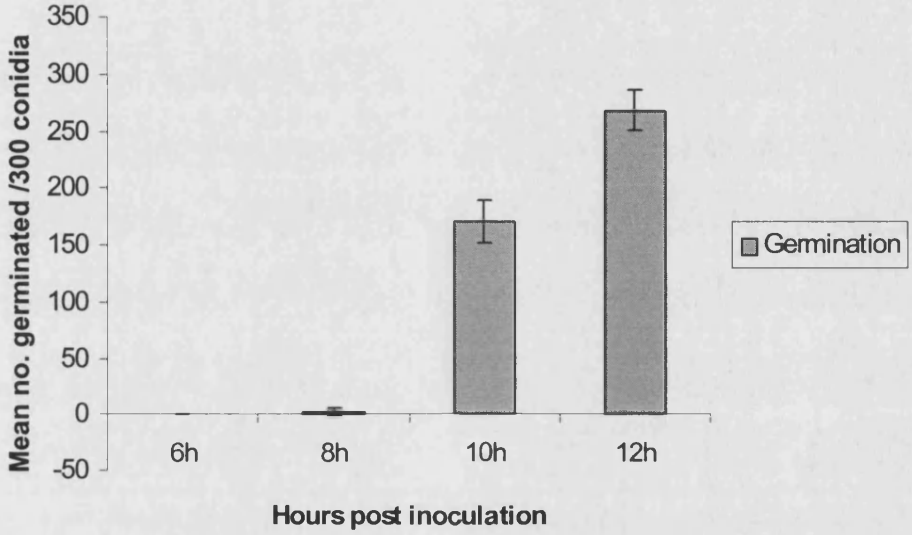


Figure 3.6: Pattern of germination of *M.anisopliae* var. *acridum* on non-sterilised *S.gregaria* hind wings (mean ± sd, n = 25)

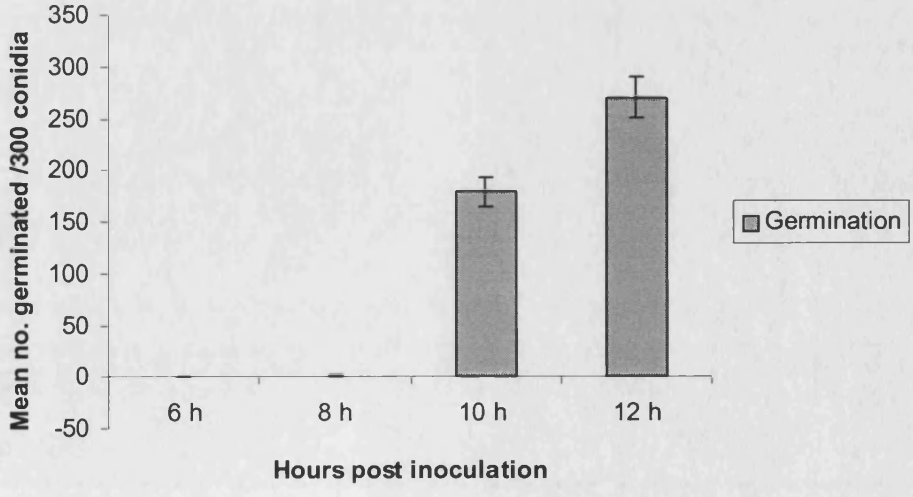
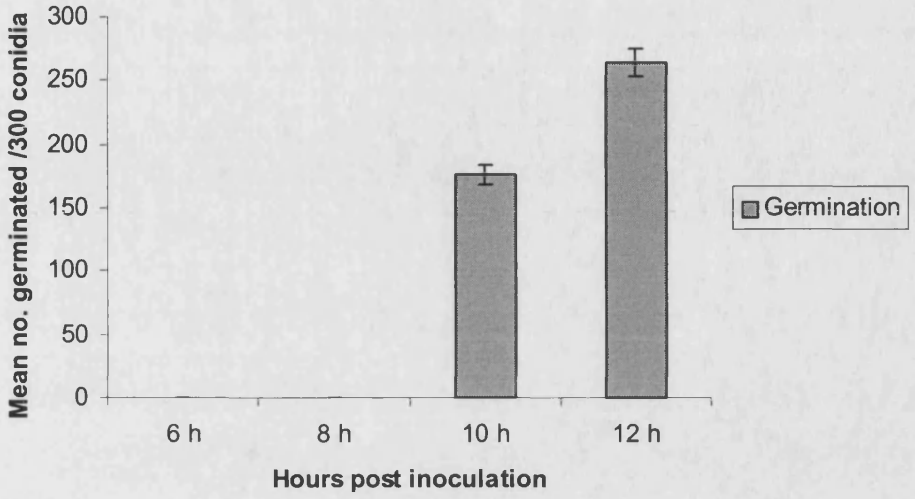


Figure 3.7: Pattern of germination of *M.anisopliae* var. *acridum* on *S.gregaria* hind wings from *S.gregaria* reared in axenic conditions (mean ± sd, n = 25)



Appressoria formation

Figures 3.8, 3.9 and 3.10 show the appressoria formation on sterilised, non-sterilised and axenic desert locust hind wing treatments respectively.

In contrast to germination the microbial status of the locust wings significantly affected appressorium formation. With chemically sterilised and non-sterilised wings, terminal appressoria first appeared at 12-18 h, and by 30 h 135.6 ± 4.6 and 136.4 ± 5.7 out of 300 (ca. 47%) conidia had germ tubes with appressoria on sterilised and non-sterilised hind wings respectively.

Appressoria formation on axenic wings, however, had a different pattern. In this case appressoria did not appear until 18-24 h. At both 24 h and 30 h the number of appressoria per 300 conidia was significantly less on axenic wings than on wings of the other two treatments ($p<0.01$, Kruskal Wallis test). Therefore, appressoria

formation appears to be impeded on axenic wings, when compared to chemically sterilised and non-sterilised treatments.

There were no significant differences between appressorial formation on sterile wings and ¼ SDA ($p<0.05$, Mann-Whitney U test).

Figure 3.8: Appressoria formation of *M.anisopliae* var. *acridum* on sterilised *S.gregaria* hind wings (mean ± sd, n = 25)

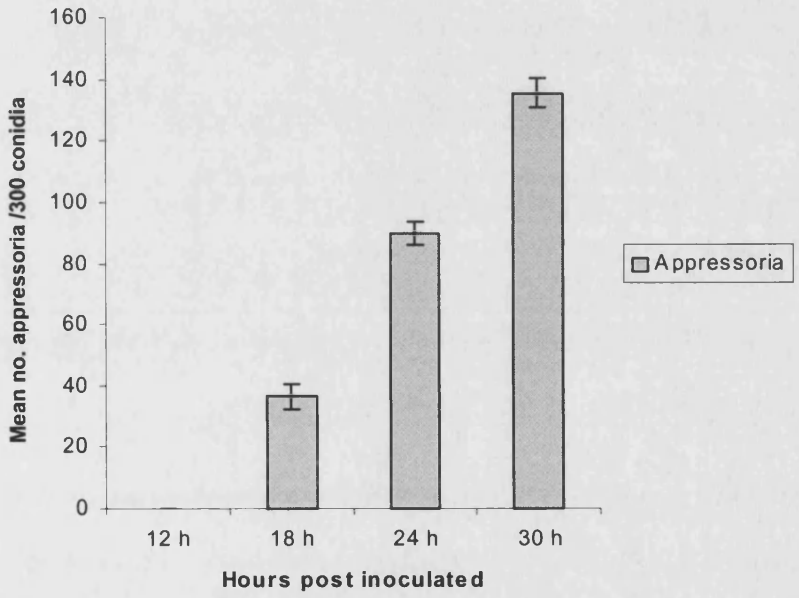


Figure 3.9: Appressoria formation of *M.anisopliae* var. *acridum* on non-sterilised *S.gregaria* hind wings (mean ± sd, n =25)

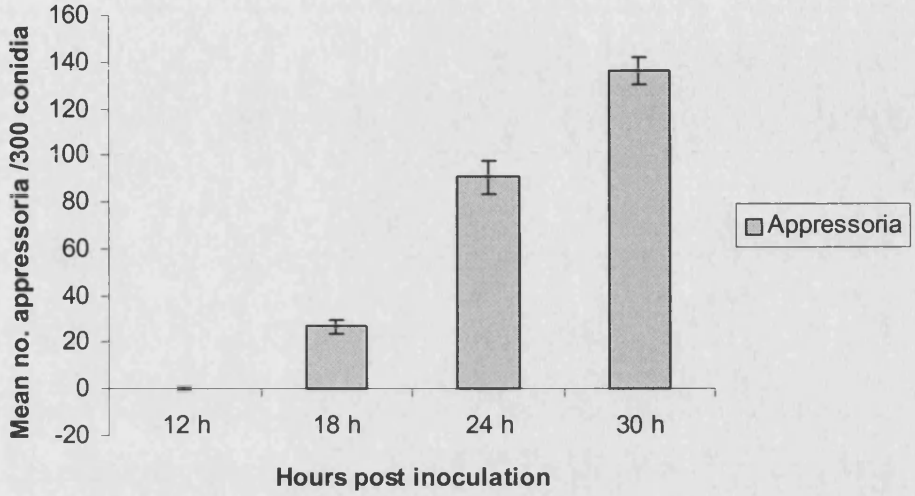
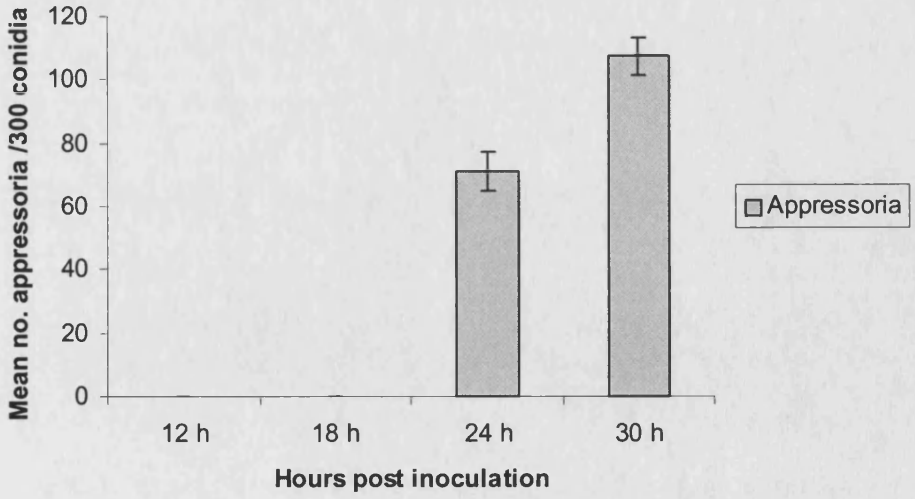


Figure 3.10: Appressoria formation of *M.anisopliae* var. *acridum* on *S.gregaria* hind wings reared under axenic conditions (mean ± sd, n = 25)



3.1.5 Germination and appressoria formation on hind wings from other insect Orders

Metarhizium anisopliae var. *acridum* isolate 330189 is specific for certain species in the Order Orthoptera. The basis of this specificity is unknown. It is possible that host range of this isolate is at least in part due to a requirement for specific nutrients or signalling molecules to initiate germination/appressoria formation. This was investigated, using the blowfly, *Calliphora vomitoria* (Order: Diptera), and the flour beetle, *Tenebrio molitor* (Order: Coleoptera).

Germination

Results of germination studies on the wings of the blowfly and flour beetle are shown in Figures 3.11 and 3.12 respectively. Germination was significantly slower and less extensive on both the wings of *C.vomitoria* and *T.molitor* than on sterile and non-sterile wings of the desert locust ($p<0.01$, Kruskal Wallis test). At 18 h post inoculation only 19.8 ± 1.3 out of 300 conidia had germinated on *C.vomitoria* wings, and this increased to just 39.4 ± 5.2 at 24 h post inoculation, whereas 170.2 ± 19 per 300 conidia had germinated by 12 h post inoculation on sterile locust wings. A similar poor performance was evident on *T.molitor* wings.

Figure 3.11: Germination of *M.anisopliae* var. *acridum* on *C.vomitoria* hind wings (mean ± sd, n = 75)

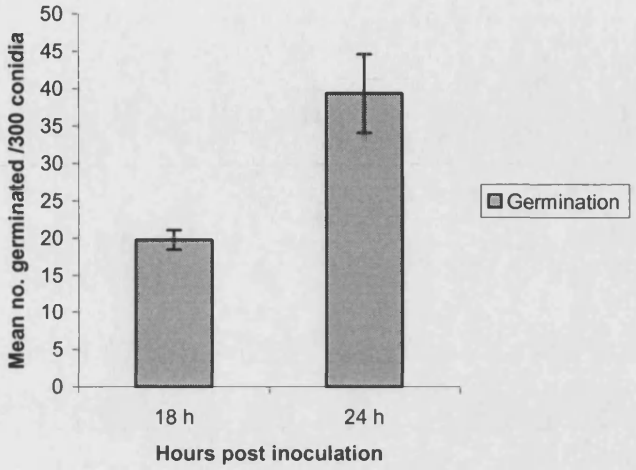
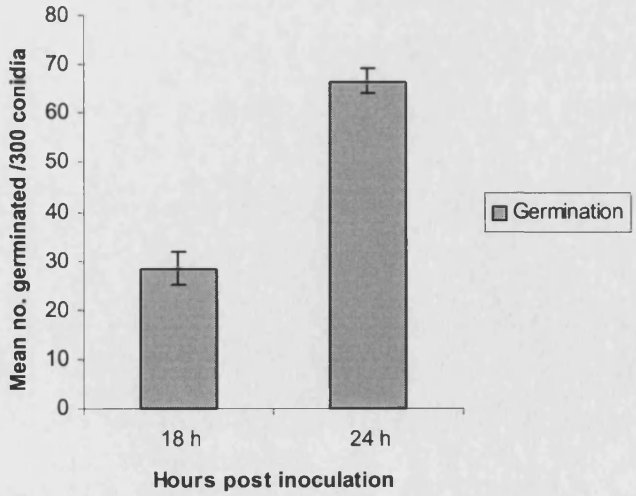


Figure 3.12: Germination of *M.anisopliae* var. *acridum* on *T.molitor* hind wings (mean ± sd, n = 75)



Appressoria formation was lower on wings excised from *C.vomitoria* (~ 20% conidia had appressoria, Fig. 3.13) than on sterilised locust wings (~ 33%, Fig. 3.8) at 24h post inoculation. Appresoria formation on *T.molitor wings*, however, was very much greater (89%, Fig. 3.14) than on sterilised locust hind wings despite lower germination levels on *T.molitor* wings.

Figure 3.13: Appressoria formation of *M.anisopliae* var. *acridum* on *C.vomitoria* hind wings (n = 75)

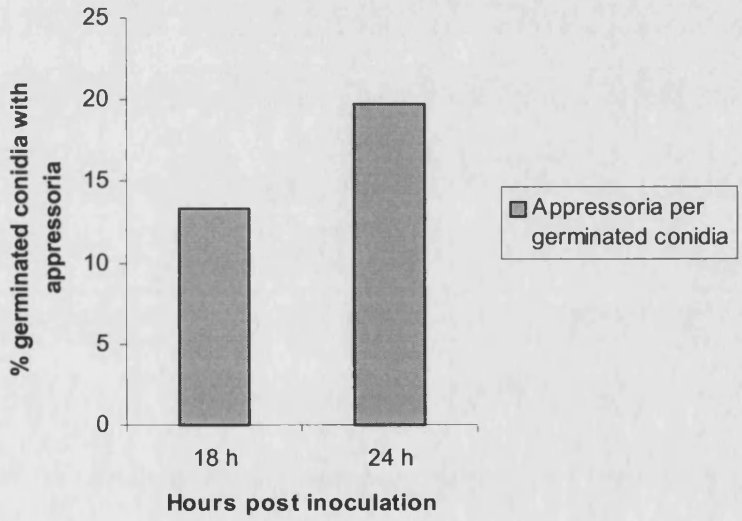
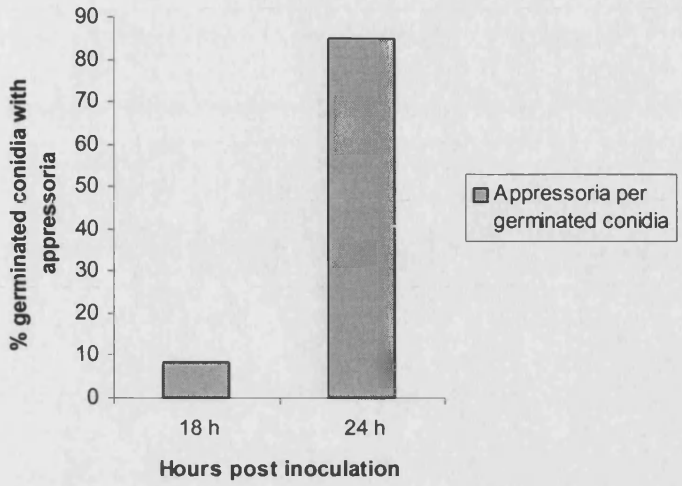


Figure 3.14: Appresoria formation of *M.anisopliae* var. *acridum* on *T.molitor* hind wings (n = 75)



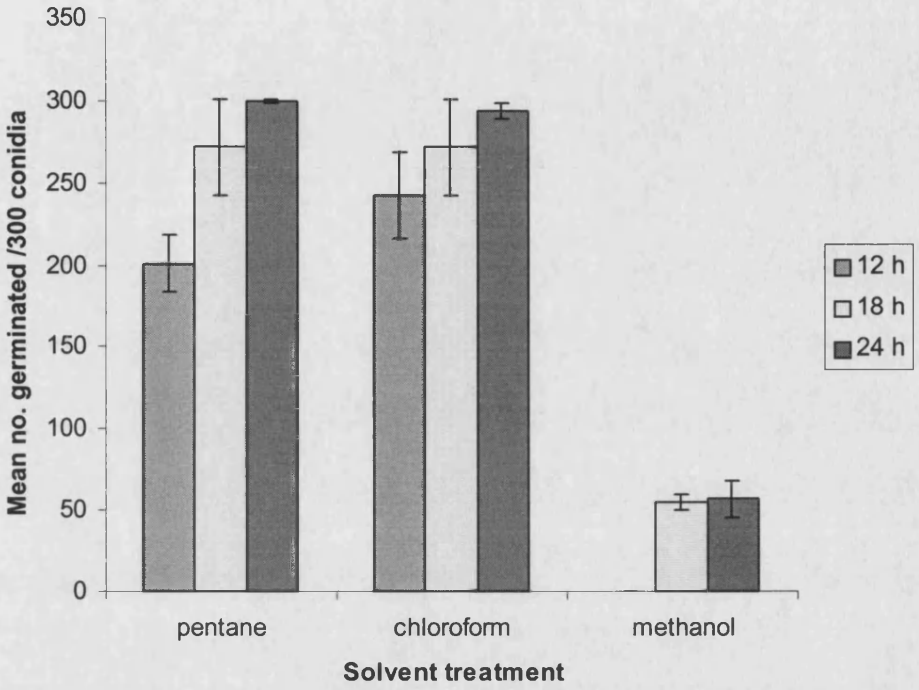
3.1.6 Germination and appressoria formation on extracted *S.gregaria* hind wings

This experiment looked at the effect of removing different elements of the epicuticle of the desert locust hind wing with solvents on the ability of *M.anisopliae* var. *acridum* conidia to germinate. Hind wings were extracted in pentane, chloroform and methanol solvents in succession and then dried and inoculated with the fungus. The results of the experiments are summarised in figures 3.15 and 3.16.

Germination

Very good levels of germination occurred on wings that had been treated with pentane and chloroform. Values were very similar to those recorded at similar time periods post inoculation on non-treated, sterilised locust hind wings. However, germination was significantly reduced on the methanol extracted hind wings. There was no germination at all 12 h post inoculation and had only reached a mean of 55 ± 4.5 of 300 conidia by18 h post inoculation.

Figure 3.15: Germination at various time intervals of *M.anisopliae* var. *acridum* on *S.gregaria* hind wings extracted in solvents (mean ± sd, n = 75)



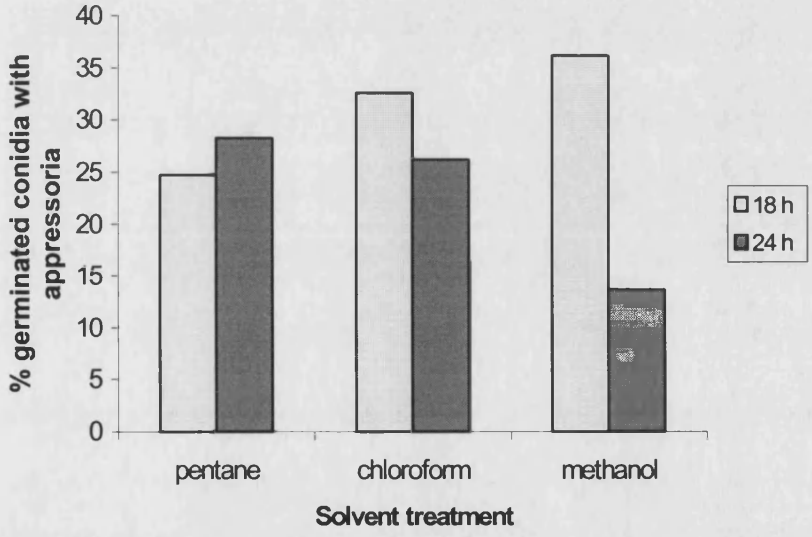
The differences between germination on methanol extracted wings and pentane and chloroform extracted wings were highly significant ($p<0.01$, Kruskal Wallis test).

Appressoria formation

The investigation was extended to examine appressoria formation on extracted desert locust hind wings. At 18 h post inoculation 25% of conidia had appressoria on pentane extracted hind wings, ~34% conidia had appressoria on wings extracted with chloroform, but almost 40% conidia had appressoria on methanol extracted wings. Appressoria formation on pentane and chloroform extracted hind wings was higher at 18 h inoculation was greater than that found at 18 h post inoculation on standard

sterilised hind wings (a mean of 36.6 ± 4.1 per 300 conidia, figure 3.5). Appressoria formation on the methanol extracted hind wing preparation was very much higher than mean appressoria formation on standard sterilised hind wings at 18 h post inoculation. The situation was reversed at 24 h as appressoria formation fell to less than 15% conidia with appressoria on methanol treated hind wings, compared to ~45% on desert locust wings.

Figure 3.16: Appressoria formation of *M.anisopliae* var. *acridum* at various time intervals on *S.gregaria* hind wings extracted in various solvents (n = 25)



3.2.0 Removal of germinated *Metarhizium anisopliae* var. *acridum* conidia

3.2.1 Removal of *M.anisopliae* var. *acridum* germlings from glass microscope slides

The aim of this experiment was to develop a protocol, which would allow removal of germinated *M.anisopliae* var. *acridum* conidia from wings, without damaging the chemical or physical structure of the surface. A physical method (a controlled, high-

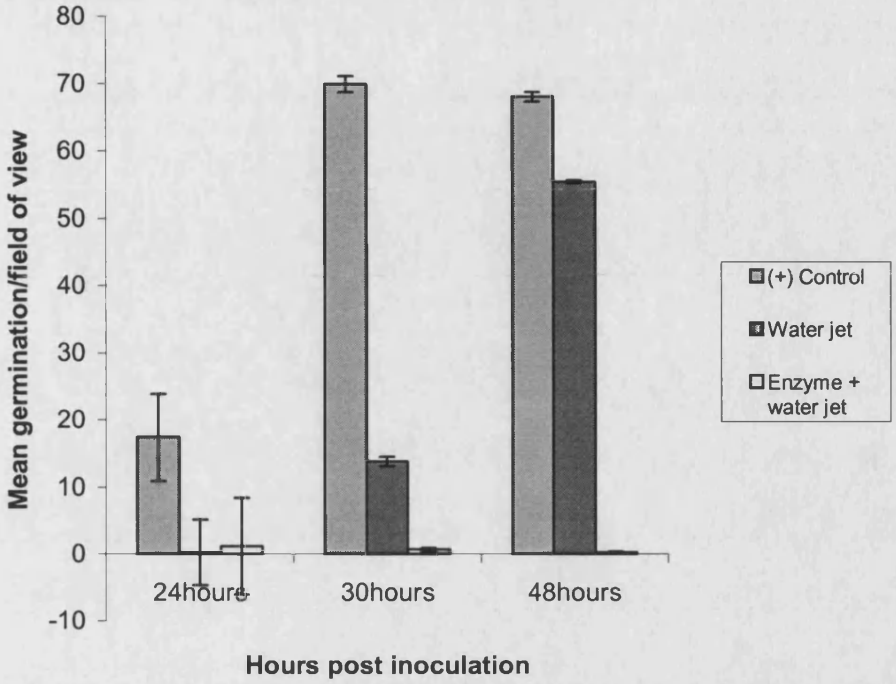
pressure water jet) was compared with a biochemical method (laminarinase enzyme coupled with a water jet).

Laminarinase attacks β 1, 3 glucans, which appear to be a major component in the extracellular mucus adhesive secreted by the fungus during germination (unpublished). This adhesive aids in attachment of the conidium and germ tube during germination and is evident in Cryo-SEM pictures of germinating conidia (see Fig.3.38, Plate Two in section 3.5.1 of this chapter).

Tests were conducted first on glass slides. Conidia were allowed to germinate in a 0.0125% Yeast Extract Medium solution for 24, 30 and 48 h, then subjected to 2 treatment regimes in an attempt to remove them.

At 24 h post inoculation, mean germination on the control glass slide preparation was 17.45 ± 6.5 per 100 conidia, which fell to 0.3 ± 1.2 after water jet treatment and 1.2 ± 0.7 after enzyme with water jet treatment. At 30 h and 48h post-inoculation, when control germination was high the water jet had progressively less effect. By 48 h enzyme treatment was necessary to remove a substantial number of germlings, presumably due to the increased amount of mucus present at this time. At 30 h and 48 h differences between treatments were highly significant ($p<0.01$, Kruskal Wallis test).

Figure 3.17: Removal of *M.anisopliae* var. *acridum* germlings from the surface of glass slides using water jet only and laminarinase + water jet treatments (mean ± sd)

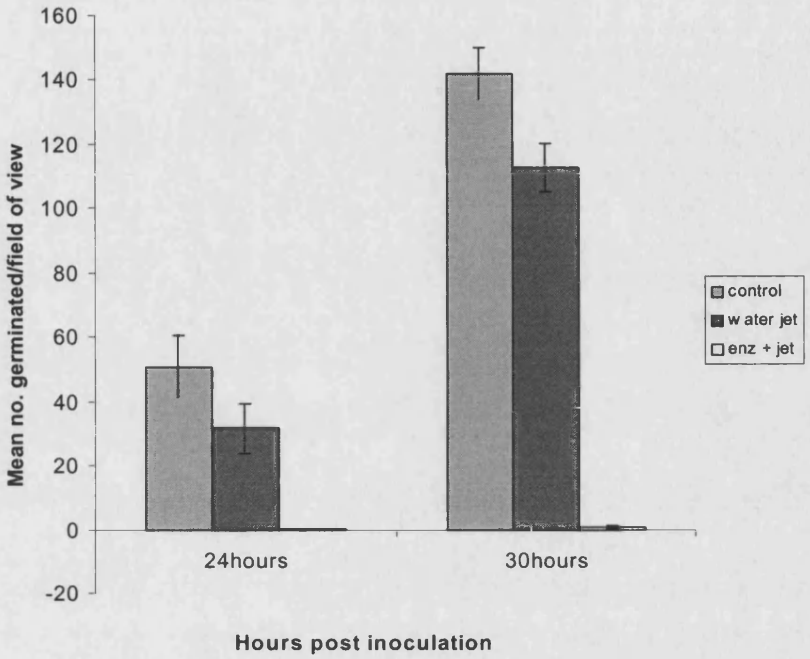


3.2.2 Removal of *M.anisopliae* var. *acridum* germlings from the surface of excised *S.gregaria* hind wings

The previous experiment established that the combination of laminarinase enzyme with a controlled water jet was the most effective method of removal of germlings from the surface of a glass microscope slide. The technique was then applied to the removal of germlings from the surface of desert locust hind wings.

Figure 3.18 shows the outcome of this experiment. At 24 h post inoculation a control mean germination of 50.9 ± 6.5 was recorded per field of view of a hind wing. Following treatment of the hind wings with a controlled water jet, the mean germination fell to 31.6 ± 4.7 conidia per field of view. The germination after enzyme with water jet treatment was 0.13 ± 0.1 per field of view.

Figure 3.18: Removal of *M.anisopliae* var. *acridum* germlings from the surface of excised *S.gregaria* hind wings, using water jet only and laminarinase + water jet treatments (mean ± sd)



At 30 h post inoculation, (a time after the appearance of appressoria but before penetration peg formation, as penetration pegs might anchor the fungus and produce other changes to the cuticle) the highest mean germination of 142 ± 4.5 per field of view occurred on the control preparation. Mean germination on the hind wings after treatment with the controlled water jet was 113 ± 6.2 per field of view. After enzyme plus water jet treatment there were very few germinating conidia remaining, giving a mean of 0.53 ± 0.1, suggesting almost complete removal of germlings by the enzyme and water jet method.

The Kruskal Wallis test was applied to the data to demonstrate that the differences between the treatments were significant ($p<0.01$).

This experiment established an extremely effective protocol for removal of nearly 100% of conidia from the desert locust hind wings. This was further confirmed as a highly efficient method through Cryo-SEM studies of the wing before and after treatment with laminarinase and a water jet (Figures 3.41 and 3.42, more detail of which is given in section 3.5.1 of this chapter).

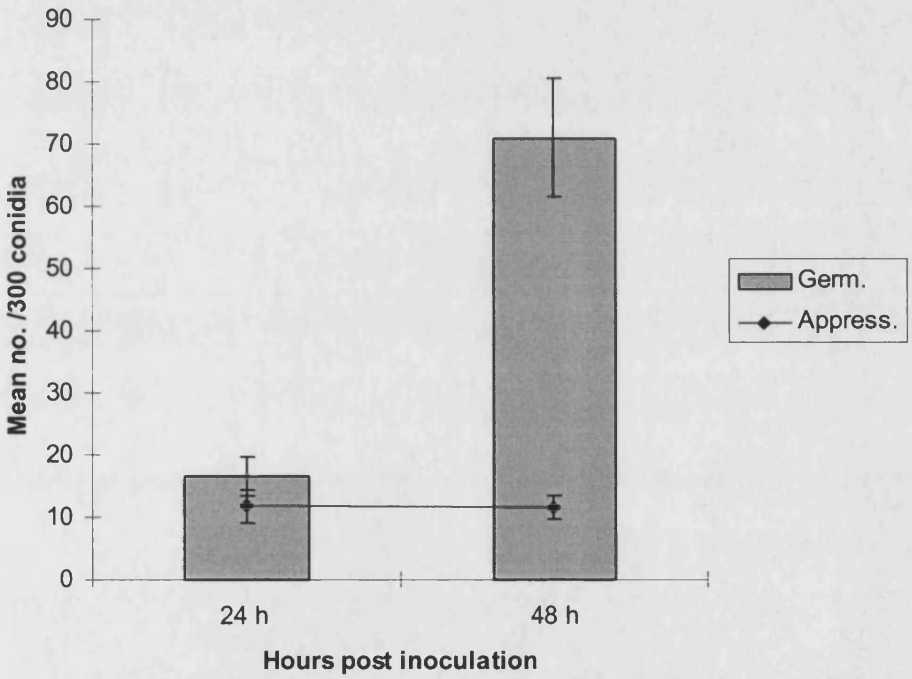
3.2.3 Germination and appressoria formation on excised *S.gregaria* hind wings previously attacked by fungus

This experiment was performed with the aim of establishing the extent to which available nutrients on the epicuticle of a desert locust hind wing are depleted by germination and germ tube formation of a 4 x 10^6 conidia ml^{-1} suspension of *M.anisopliae* var. *acridum*. This was investigated by inoculating desert locust hind wings that had been treated with *M.anisopliae* var. *acridum* then subsequently removed with laminarinase/water jet treatment. Standard sterilised desert locust hind wings were used for comparison (Figures 3.4 and 3.5).

The results of this experiment are shown as Figure 3.19. At 24 h post inoculation mean germination per 300 conidia was just 16.6 ± 3.2, and this had increased to 71 ± 9.5 by 48h. A comparison with standard sterilised desert locust wings (mean germination of 268.6 ± 17.5 per 300 conidia at 12 h post inoculation) shows greatly reduced germination.

The depletion of nutrients by prior fungal treatment had an even more dramatic effect on appressoria formation. Indeed at 11.8 ± 2.6 per 300 conidia at 30 h and 11.6 ± 1.8 per 300 at 48 h post inoculation, there appeared to be a lack of stimulation of appressoria formation. This poor performance is in contrast to the 107.4 ± 5.7 appressoria per 300 conidia at 30 h post inoculation obtained on standard sterilised desert locust hind wings.

Figure 3.19: germination and appressoria formation of *M.anisopliae* var. *acridum* conidia on *S.gregaria* hind wings previously attacked by fungus (mean ± sd, n = 25)



3.3.0 Analysis of extracts from excised desert locust (*Schistocerca gregaria*) hind wings

3.3.1 Contents of pentane extracts from *S.gregaria* hind wings, pre- and post-fungus (GC-MS analysis)

Figure 3.20 shows a typical total ion current (TIC) gas chromatogram produced by GC-MS analysis of the pentane extract taken from untreated desert locust hind wings.

The series of peaks produced were identified from their mass spectra and through the use of the Hewlett Packard Chemstation Wiley 275.1 database, and listed in Table 3.1. Six of the seven peaks were identified as hydrocarbons, specifically *n*-alkanes. The remaining peak was identified as cholesterol.

Figure 3.21 shows a typical chromatogram of the pentane extract, obtained after inoculation of the wings with *M.anisopliae* var. *acridum.* There were 4 peaks produced, which were identified as described above. The peak identities are listed in Table 3.1. Peaks identified as pentacosane, tetracontane (*n*-alkanes) and cholesterol are not present on this post-fungus chromatogram, although they were present in the previous, pre-fungus GC-MS analysis of the pentane extract.

Figure 3.20: Typical chromatogram produced by GC-MS of a pentane extract of *S.gregaria* hind wings

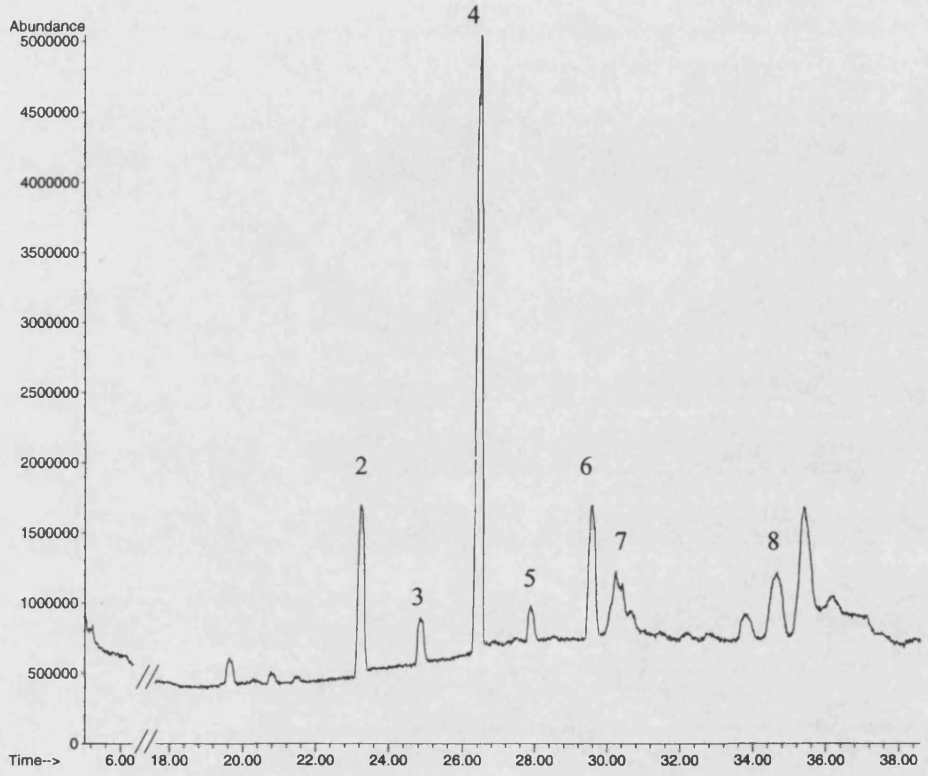


Figure 3.21: Typical chromatogram produced by GC-MS of pentane extract of *S.gregaria* hind wings after a 24h inoculation with *M.anisopliae* var. *acridum*

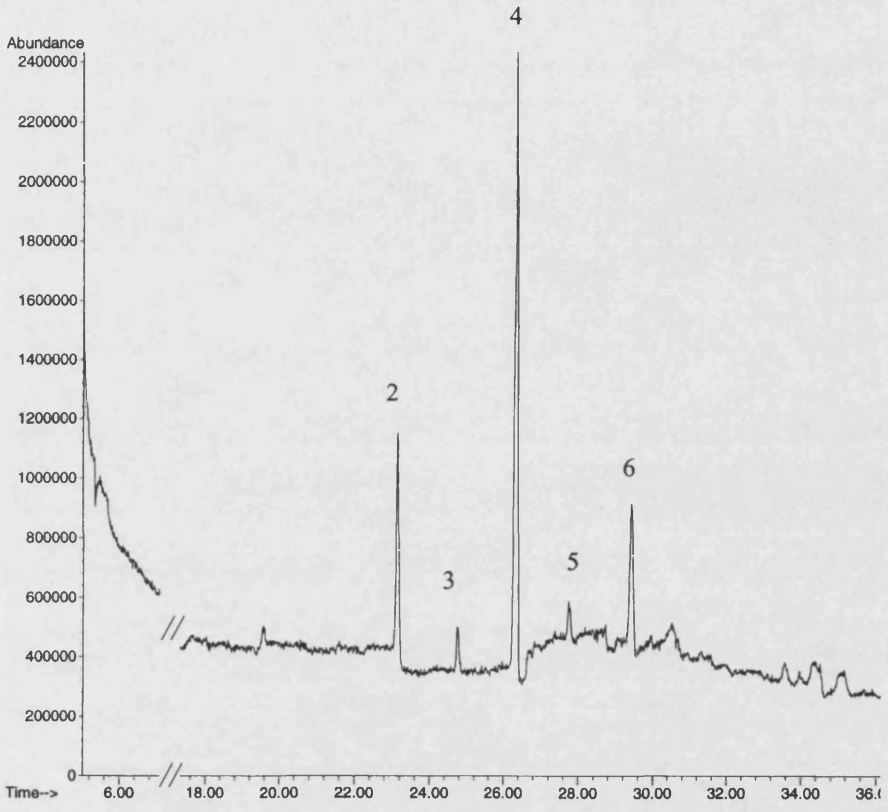


Table 3.1: Compounds present in a pentane extract of excised *S.gregaria* hind wings before and after fungal attack

Compounds present	
Pre-fungus	**Post-fungus**
1. Pentacosane	1. –
2. Heptacosane	2. Heptacosane
3. Octacosane	3. Octacosane
4. Nonacosane	4. Nonacosane
5. Triacontane	5. Triacontane
6. Hentriacontane	6. Hentriacontane
7. Cholesterol	7. –
8. Tetracontane	8. -

Figure 3.20 and 3.21 show a lower number of compounds present in the pentane extract after inoculation with *M.anisopliae* var. *acridum.* The arbitrary abundance scale on the chromatograms (y-axis) suggests that the amounts of the compounds present were reduced after inoculation with *M.anisopliae* var. *acridum.* A more detailed analysis of the quantitative effects of fungal growth on wing lipids was carried out using octacosane as a representative hydrocarbon, (see section 3.3.4).

3.3.2 Contents of chloroform extracts from *S.gregaria* hind wings, pre- and post-fungus (GC-MS analysis)

A typical TIC gas chromatogram produced by analysis of the chloroform extract of untreated desert locust hind wings is presented in Figure 3.22 and Table 3.2 shows the compounds identified from their mass spectra and the Wiley database from the

chromatogram. The chloroform extract was composed primarily of fatty acid esters (16 and 18 carbon compounds) and *n*-alkanes (25-29 carbon compounds).

Table 3.2: Compounds present in chloroform extract of *S.gregaria* hind wings pre- and post-fungal attack

Compounds present	
Pre-fungus	**Post-fungus**
1. Hexadecanoic acid, methyl ester	
2. 9,12 Octadecanoic acid, methyl ester	
3. Octadecanoic acid, methyl ester	No identified peaks
4. Pentacosane	
5. Heptacosane	
6. Nonacosane	

Figure 3.22(b) shows a typical chromatogram produced for the chloroform extract made from hind wings after inoculation with *M.anisopliae* var. *acridum* for 24 h. Although some peaks were present on the chromatogram, accurate identification was not achieved using the Chemstation Wiley database or mass spectra data. Therefore no identifiable compounds were recorded as being present in GC-MS analysis of the post-fungus chloroform wing extract.

Figure 3.22(a): Typical chromatogram produced by GC-MS of a chloroform extract of *S.gregaria* hind wings

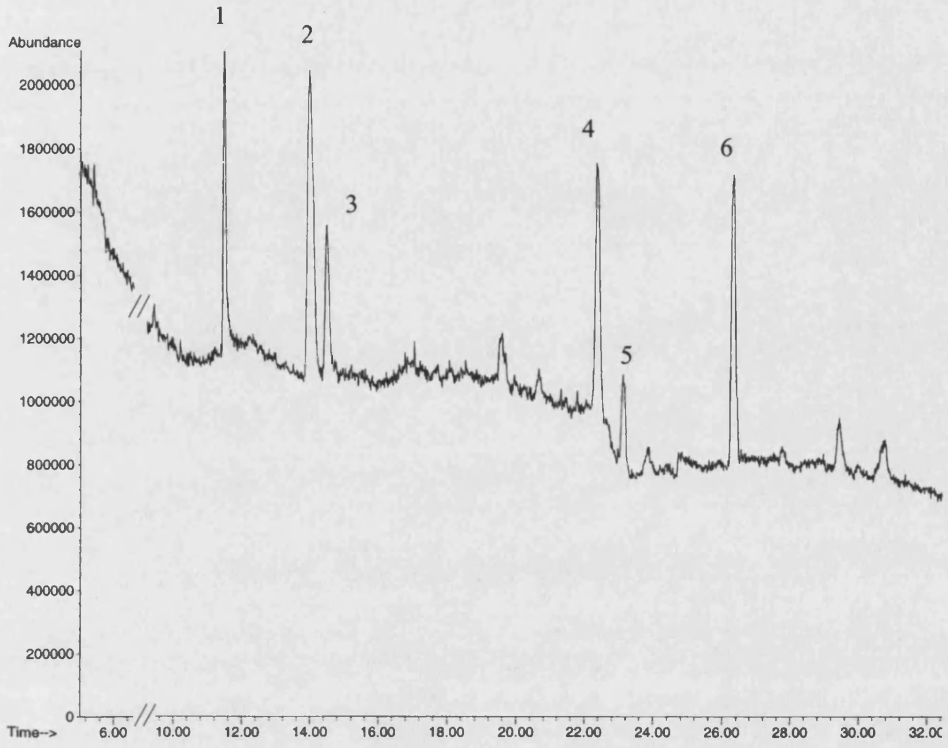
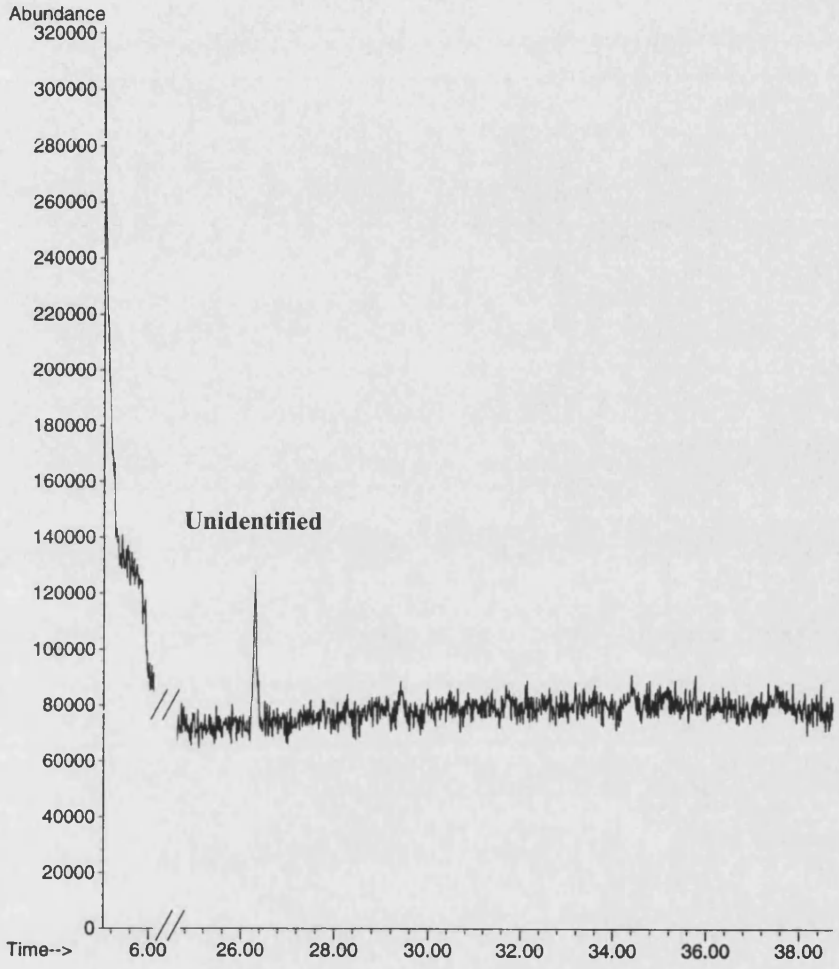


Figure 3.22(b): Typical chromatogram produced by GC-MS of chloroform extract of *S.gregaria* hind wings after a 24h inoculation with *M.anisopliae* var. *acridum*



3.3.3. Contents of methanol extracts from *S.gregaria* hind wings, pre- and post-fungus (GC-MS analysis)

The methanol extract from untreated desert locust hind wings was derivatised with Methyl-8 concentrate to allow detection of any fatty acids in the extract. Table 3.3 summarises the content of the pre-fungus methanol extract, and a typical TIC chromatogram is shown in Figure 3.23

Figure 3.23: Typical chromatography produced by GC-MS of a methanol extract of *S.gregaria* hind wings

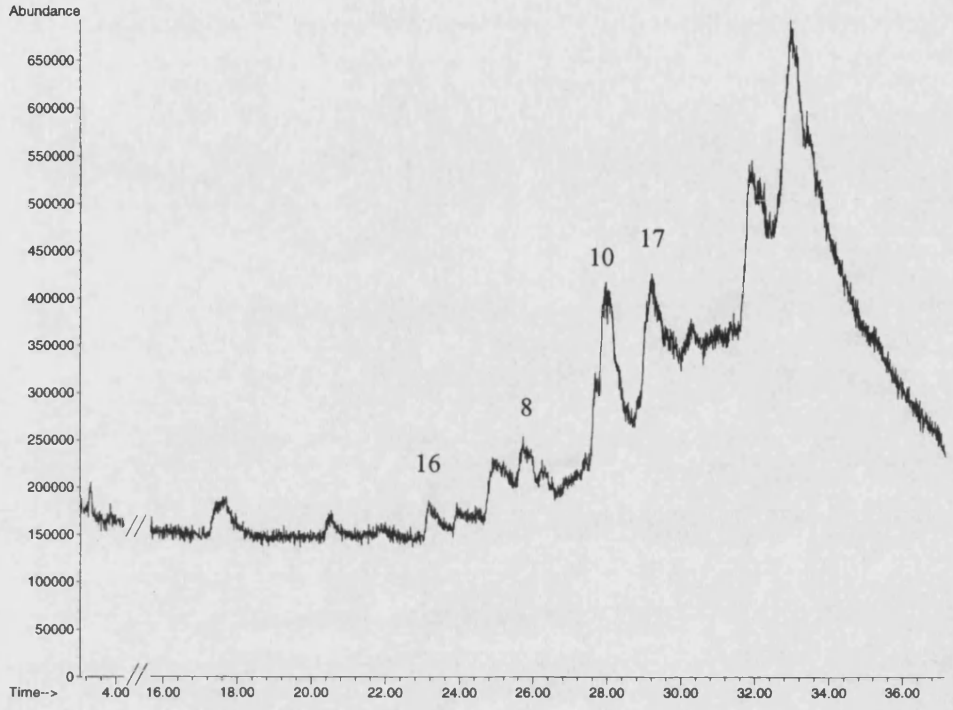


Figure 3.24: Typical chromatogram produced by GC-MS of methanol extract of *S.gregaria* hind wings after a 24h inoculation with *M.anisopliae* var. *acridum*

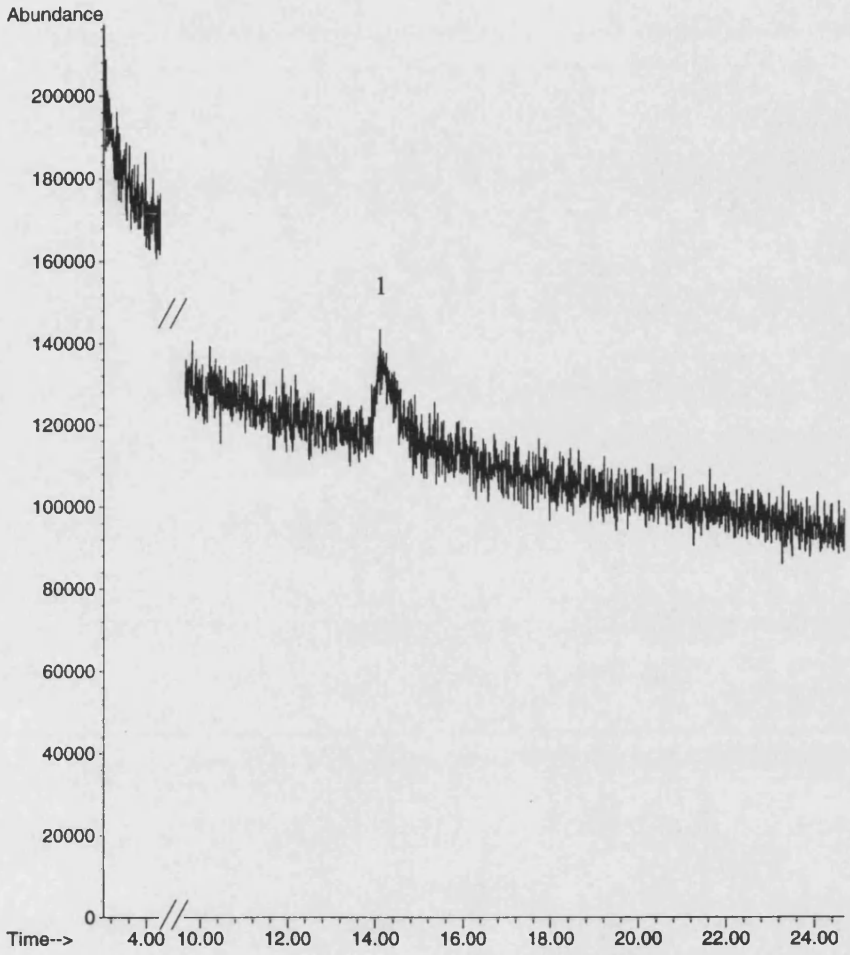


Table 3.3: Compounds present in methanol extract of excised *S.gregaria* hind wings pre- and post fungal attack

Compounds present

Pre-fungus	**Post-fungus**
1. Ethyl decanoate	N-[2-methyl propyl] 9, octadecenamide
2. Tetradecanoic acid	
3. Methyl pentadecanoate	
4. Pentadecane	
5. Hexadecanoic acid	
6. Unidentified	
7. 3-methyl heptadecane	
8. 1-heptadecane	
9. [9-methyl] heptadecanoic acid,	
10. Octadecane	
11. Ethyl-2-hydroxy-9-octadecenoate	
12. 3, 7-dimethyl-2,6 octadecadienoic acid,	
13. 9,12 –octadecadienoic acid	
14. Eicosanoic acid	
15. 3-methyl heneicosane	
16. 2-methyl tricosane	
17. Tricosane	

Figure 3.24 shows a typical post-fungus TIC chromatogram, which featured only one peak. This peak was not present in the pre-fungus chromatogram for the methanol extract. The compound, N-[2-methyl propyl] 9, octadecenamide (Table 3.3) remained after 24 h inoculation of desert locust wings with *M.anisopliae* var. *acridum* and was not identified as one of the compounds present in the pre-fungus methanol extract. This sample was also derivatised with Methyl-8 concentrate prior to analysis.

3.3.4. Quantification of octacosane – pre- and post-fungus

Octacosane, a long chain *n*-alkane present in the desert locust wings pentane extract, was quantified before and after inoculation for 24 h with *M.anisopliae* var. *acridum.* An octacosane calibration curve was constructed through GC-MS analysis of a 3 standard solutions containing octacosane and an internal standard of docosane (a long-chain *n*-alkane which is not present in the pentane extract). Figures 3.25(a), (b) and (c) show the chromatograms used to construct the calibration curve. Octacosane concentations of 22.2μg ml^{-1}, 11.1μg ml^{-1} and 5.6μg ml^{-1} in hexane solvent were analysed. Docosane concentration was kept constant throughout. The peak area ratio for each n-alkane for each chromatogram were obtained through the HP ChemStation software, and the peak area ration for octacosane divided by that for docosane and a sample ratio reached for each chromatogram. These values were plotted in Minitab 11 to create the calibration graph shown in Figure 3.26

Figure 3.25: GC-MS chromatograms produced to create a calibration curve for octacosane concentration. Docosane was the internal standard

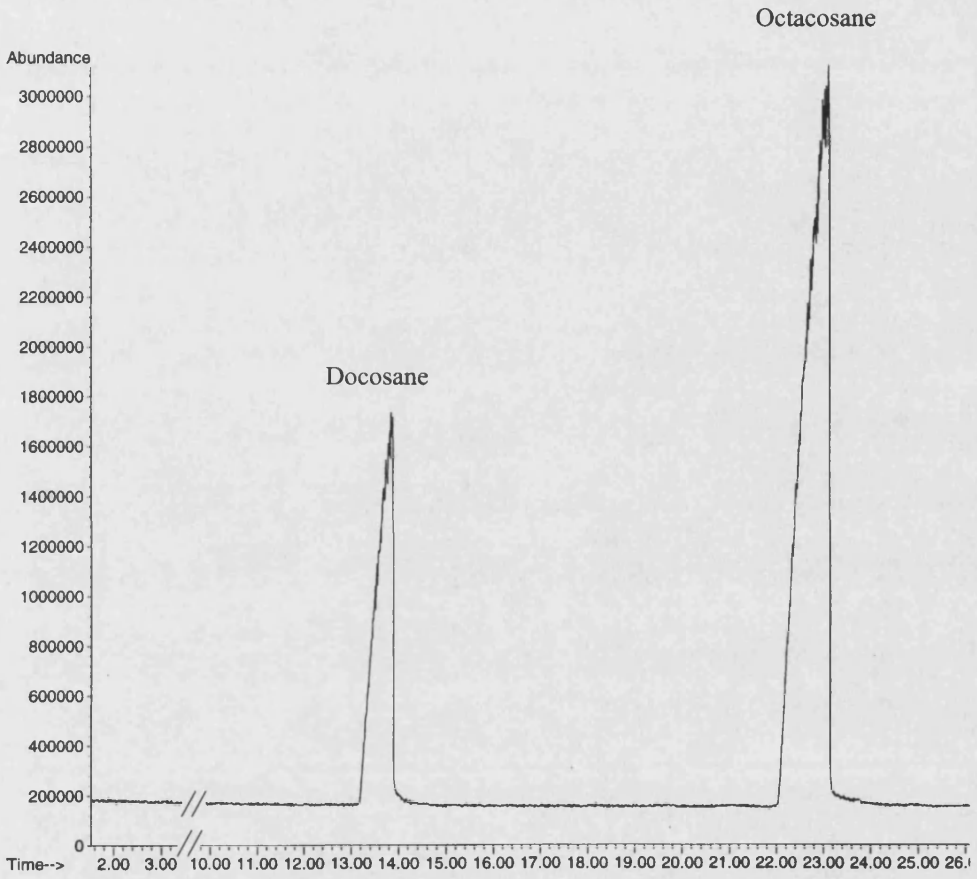


Figure 3.25 (a) Dilution 1: 200 μl Octacosane

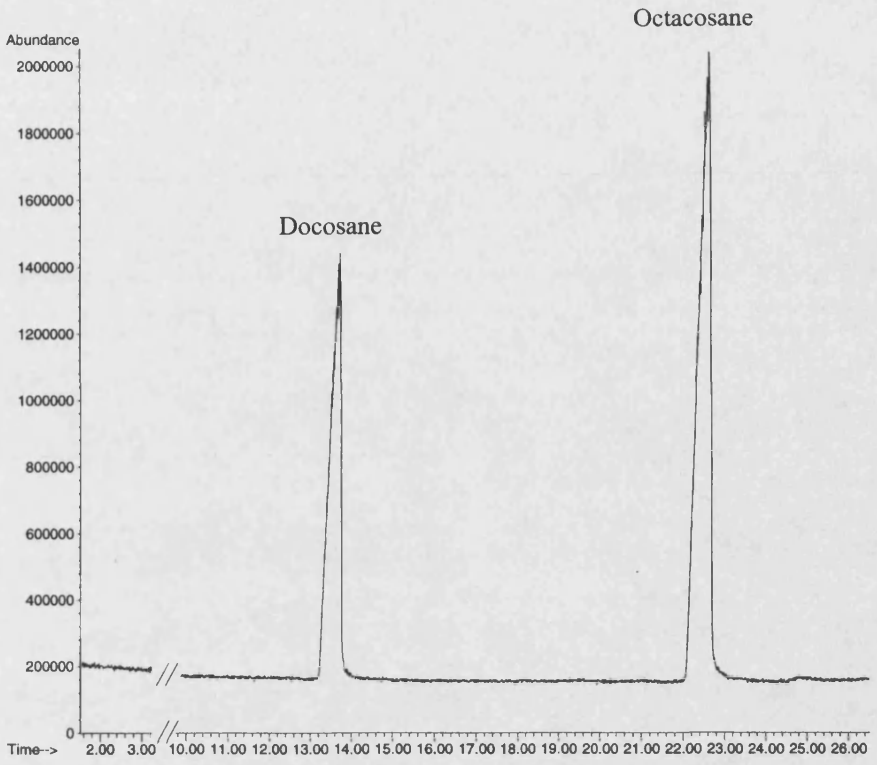


Figure 3.25 (b) Dilution 2: 100 μl Octacosane

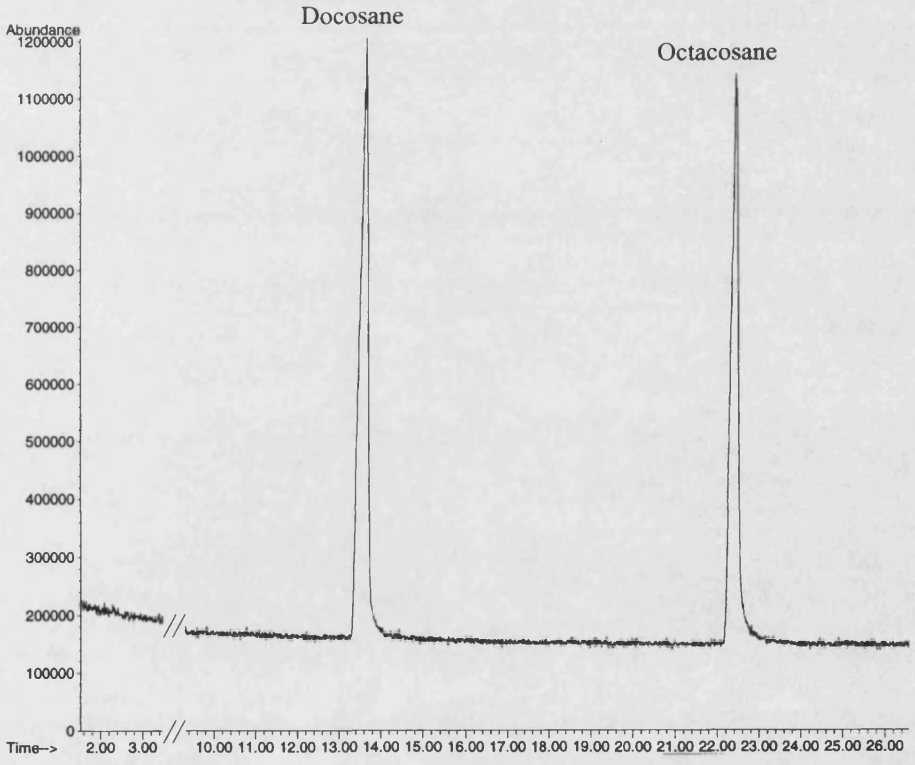


Figure 3.25 (c) Dilution 3: 50 μl Octacosane

Figure 3.26(a): Calibration curve created by Figure 3.25(a), (b) and (c)

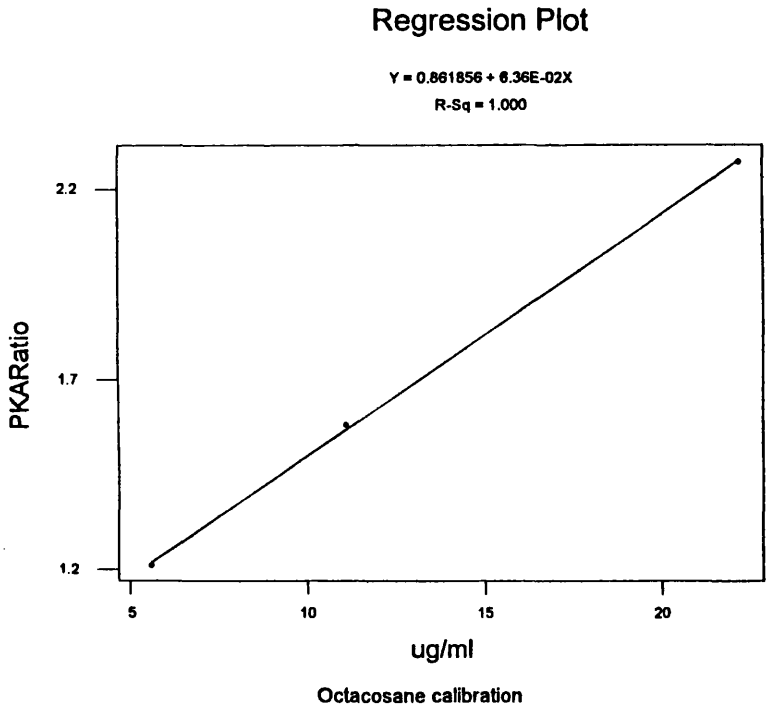


Figure 3.26(b): Pentane extract containing docosane internal standard pre-fungus

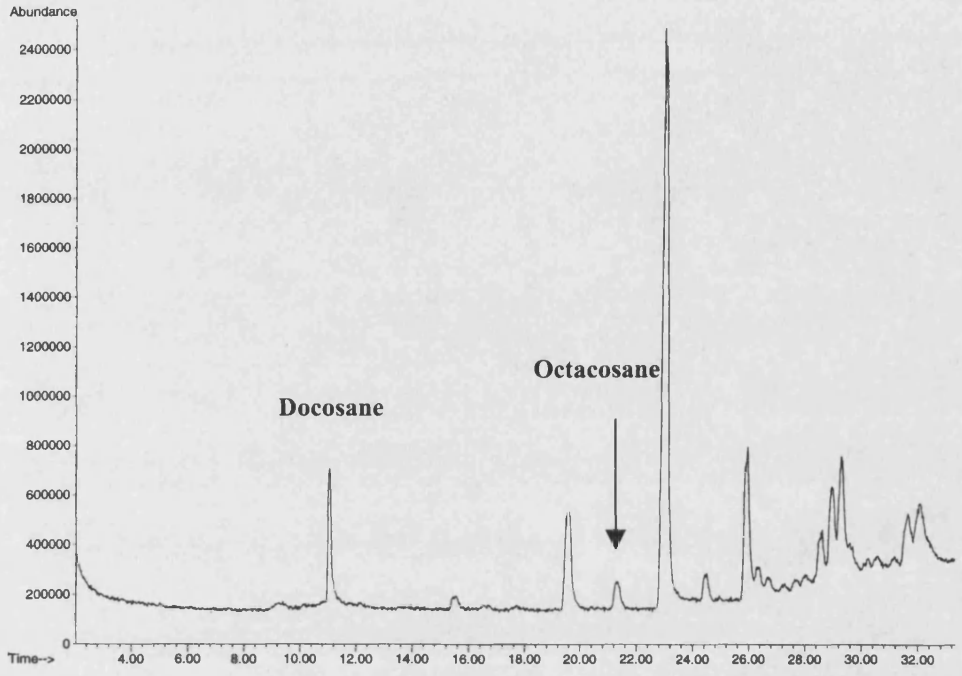
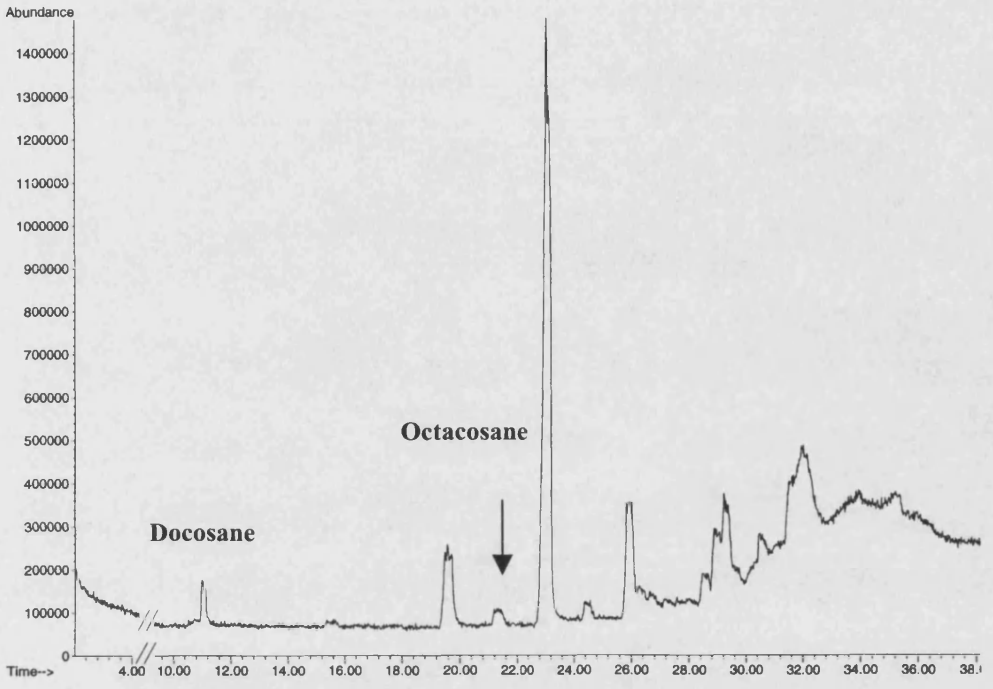


Figure 3.26(c): Pentane extract containing docosane internal standard post-fungus



Regression analysis provided an equation for the line (1, below), and the excellent R^2 value of 1.00.

Sample ratio = 0.862 + 0.0636 μg ml^{-1} (1)

(where 0.862 = intercept and 0.0636 = slope)

This equation could then be rearranged to find the number of μg ml^{-1} octacosane in a pentane extract for locust hind wings (2, below)

(Sample ratio - 0.862) ÷ 0.0636 = *X* μg ml^{-1} octacosane (2)

Pentane extracts from locust hind wings, before and after inoculation with *M.anisopliae* var. *acridum*, were prepared containing the internal standard docosane, and sample ratios for octacosane and docosane calculated.

The sample ratios for pre- and post-fungus pentane extracts allowed the calculation of the approximate amount of octacosane in the two extracts to be calculated, and these are shown in Table 3.4.

Table 3.4: Approximate content of octacosane in pentane extract from excised *S.gregaria* hind wings, before and after fungal attack

Pre-fungus octacosane $\mu g\ ml^{-1}$	Post-fungus octacosane $\mu g\ ml^{-1}$
13.9	2.27

30 wings were refluxed, and extract separated into 10 aliquots. The above figures = octacosane in 1 aliquot = ~3 wings

Considerably less octacosane was present in the fungus treated pentane extract than in the untreated extract.

3.3.5 Glucose oxidase assay on excised *S.gregaria* hind wing extracts

The estimated glucose content of desert locust wing solvent extracts, before and after inoculation with *M.anisopliae* var. *acridum*, is shown in Table 3.5. Glucose levels were determined through the glucose oxidase assay.

The pre-fungus methanol extract was the only extract which had a recordable level of glucose present (0.13mM). After inoculation with *M.anisopliae* var. *acridum* this concentration had decreased to undetectable levels.

Table 3.5: Glucose content of solvent extracts from desert locust hind wings, before and after inoculation with *M.anisopliae* var. *acridum*

Glucose content		
Treatment	**Mean O.D at 520nm** Standard wing	*Aged controls
Pentane	0.000	0.00
Chloroform	0.021	0.019
Methanol	**0.124 (= 0.13mM)**	0.130
Pentane (post fungus)	0.002	
Chloroform (post fungus)	0.001	
Methanol (post fungus)	0.024	

*An extra control preparation was incorporated into this experiment. Aged hind wing pre-fungus extracts were made from hind wings that had been excised from *S.gregaria* 24 h prior to the assay. These were subjected to the same conditions as the standard pre-fungus extracts (which had been extracted immediately upon removal from the locust) to investigate as to whether cuticular lipids change with age

3.3.6 Ninhydrin assay on excised *S.gregaria* hind wing extracts

The ninhydrin assay showed the level of nitrogen-containing compounds (substantially proteins but amino acids may make a contribution) present in the solvent extracts from desert locust wings before and after inoculation with *M.anisopliae* var. *acridum*. Table 3.6 shows the results of the assay.

As with the previous study of glucose content, the methanol extract was the only extract with measurable protein content, with 0.082μmoles being detected in the pre-fungus extract. After inoculation with *M.anisopliae* var. *acridum* the protein content of the methanol was detected as the lower amount of 0.026μmoles.

Table 3.6: Protein content of solvent extracts from desert locust hind wings, before and after inoculation with *M.anisopliae* var. *acridum*

Protein content		
Treatment	**Absorbance @ 570 nm** Standard wings	*Aged control
Pentane	0.054	0.052
Chloroform	0.029	0.036
Methanol	**0.432** **(= 0.082 μmoles)**	0.379
Pentane (post fungus)	0.007	
Chloroform (post fungus)	0.01	
Methanol (post fungus)	**0.141** **(=0.026μmoles**	

*Aged control preparations - An extra control preparation was incorporated into this experiment. Aged hind wing pre-fungus extracts were made from hind wings that had been excised from *S.gregaria* 24 h prior to the assay. These were subjected to the same conditions as the standard pre-fungus extracts (which had been extracted immediately upon removal from the locust) to investigate as to whether cuticular lipids change with age

3.3.7 Amino acid analysis on methanol extracts of *S.gregaria* hind wings before and after fungal attack

Amino acid analysis was performed on the methanol extracts from desert locust hind wings before and after inoculation with *M.anisopliae* var. *acridum.* Ninhydrin assays (section 3.3.5) indicated that only the methanol extracts contained measurable amounts of nitrogen-containing compounds (and therefore amino acids and peptides/proteins).

Table 3.7 shows free amino acid content before and after fungus. The most abundant free amino acids in the pre-fungus methanol extract were glycine, alanine and glutamine. The amino acids methionine and histidine were absent from this methanol extract. Some amino acids were absent from the post-fungus methanol extract, depite being present pre-fungus. These were threonine, cysteine, tyrosine and arginine. Overall, the levels of free amino acids were substantially less in the post-fungus methanol extract than in the pre-fungus methanol extract.

Table 3.7: Free amino acid content of methanol extract of desert locust hind wing before and after inoculation with *M.anisopliae* var. *acridum*

Free amino acid content		
Amino acid	Pre-fungus (nmole/sample)	Post-fungus (nmole/sample)
Asp	3.1	0.3
Thr	11	-
Ser	12	0.5
Glu	21	0.4
Pro	18	0.2
Gly	73	0.9
Ala	29	2.0
Cys	0.1	-
Val	7.6	0.7
Met	-	-
Ile	4.3	0.4
Leu	4.6	0.8
Tyr	5.5	-
Phe	1.7	0.3
His	-	-
Lys	5.1	0.1
Arg	4.1	-

Table 3.8 shows the amino acids present in the methanol extracts (pre- and post-fungus) after hydrolysis of the samples; to allow breakdown of proteins. Levels of amino acids recorded were greater in the post-hydrolysis analysis of the methanol extracts, than in the free amino acid analysis. However, the same amino acids, glycine, alanine and glutamine proved to be the most abundant in the post-hydrolysis, pre-fungus methanol extract, as in the pre-fungus free amino acid analysis of the methanol extract.

Cysteine and histidine were absent from pre- and post-fungus methanol extracts after hydrolysis. The amino acids methionine and lysine were entirely absent in the post-fungus methanol extract, despite being present before inoculation with *M.anisopliae* var. *acridum.*

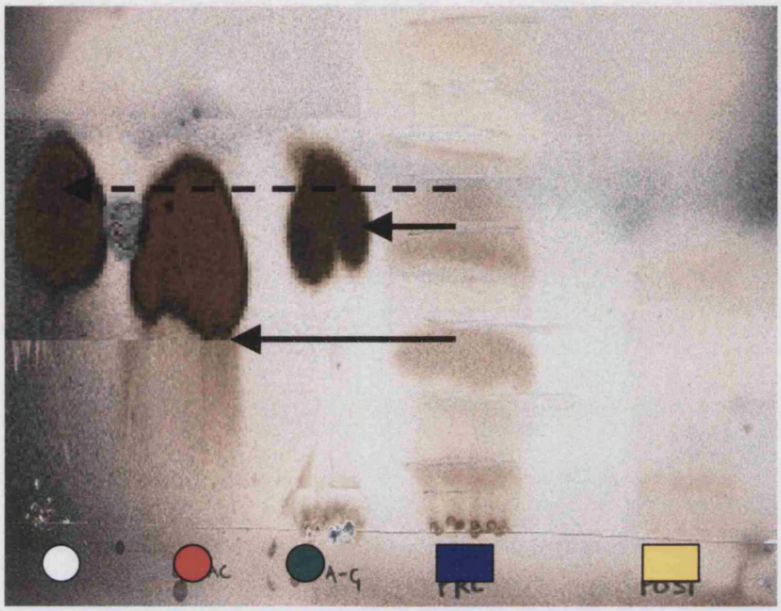
As with free amino acid analysis, the amino acid levels were substantially less in the post-fungus methanol extract then in the pre-fungus methanol extract.

Table 3.8: Post-hydrolysis amino acid content of methanol extract of desert locust hind wing before and after inoculation with *M.anisopliae* var. *acridum*

Amino acid content – post hydrolysis		
Amino acid	Pre-fungus (nmole/sample)	Post-fungus (nmole/sample)
Asp	14	0.9
Thr	9	0.7
Ser	15	1
Glu	51	1.4
Pro	41	1.5
Gly	110	4.6
Ala	95	3.6
Cys	-	-
Val	22	1.3
Met	2.3	-
Ile	12	0.8
Leu	19	1.5
Tyr	12	0.5
Phe	3.1	0.7
His	-	2.4
Lys	7.1	-
Arg	6.6	0.2

3.3.8 TLC analysis of monosaccharides in methanol extract from *S.gregaria* hind wings before and after fungal attack

Figure 3.27: TLC analysis of monosaccharides



KEY:

= Glucose	= Galactose	= *N*-A-G	= Pre-fungus	= Post-fungus

Analysis of the methanol extract of locust hind wings showed the presence of glucose (see section 3.3.5) The concentration of this sugar was drastically reduced following treatment with fungus. Thin Layer Chromatography was carried out on methanol extracts to determine whether there were other carbohydrates present in the methanol extract. Figure 3.27 above shows preliminary thin layer chromatography analysis of the methanol extracts taken from *S.gregaria* hind wings before and after inoculation with fungus for 24 hours. These methanol extracts were compared with monosccharide standards glucose, galactose and the monosaccharide amino sugar *N*-

acetyl glucosamine (is the monomer for polysaccharide chitin, found in the exoskeletons of insects).

The TLC plate above shows a large spot corresponding to N-A-G and/or glucose and a further large spot which occurs below the galactose standard in the pre-fungus extract, (see arrows). Another band occurred just above the origin. Both these unknowns suggest the presence of higher molecular weight carbohydrates. The absence of the bands post fungus treatment suggests they are used by the fungus during germination.

3.4.0 Germination of *M.anisopliae* var. *acridum* on authentic compounds *vs.* biological extracts *in vitro*

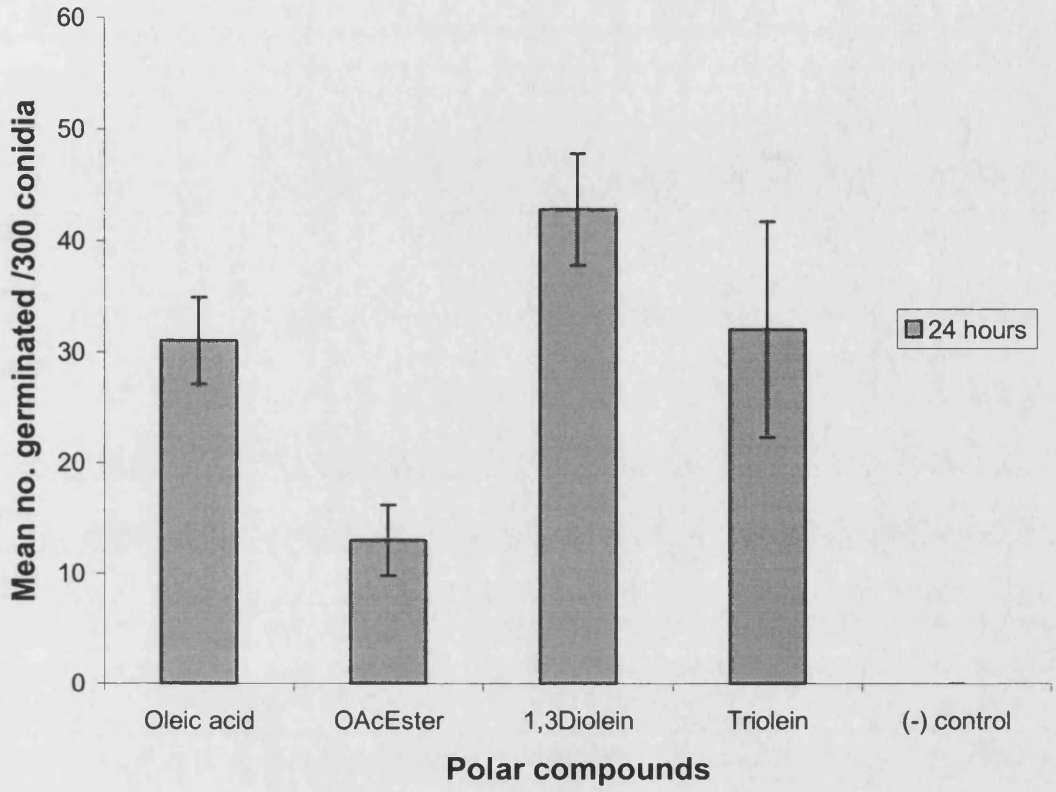
3.4.1 Germination on authentic polar lipids

A number of authentic polar lipids were selected as sole sources of carbon in a germination preference study for *M.anisopliae* var. *acridum*. Polar compounds used represented groups known to be present on the epicuticle of the desert locust, both from personal study and previously published work (Lockey and Oraha, 1990). The polar lipids tested included a free fatty acid (oleic acid), a free fatty acid ethyl ester (oleic acid ethyl ester), a diacylglycerol (1,3 diolein) and a triacylglycerol (triolein).

The compounds, as sole carbon source, were each combined with basal salts agar medium, which provided a solid substrate for germination and a nitrogen source for nutrition, then incubation at 27°C at 100% humidity.

Germination was determined 24 h after inoculation and the mean number of germinated conidia per 300 recorded. Basal salts agar was used as a control. Germination on authentic polar lipids was lower than at 16 h post inoculation on ¼ SDA (Figure 3.1) and on the standard sterilised desert locust hind wing at 12 h post inoculation (Figure 3.4), which showed almost 100% germination. The ester supported less germination than either the diacylglycerol or triacylglycerol, or the free fatty acid. The basal salts agar control elicited no germination.

Figure 3.28: Germination of *M.anisopliae* var. *acridum* on authentic polar lipids (mean ± sd, n = 5)



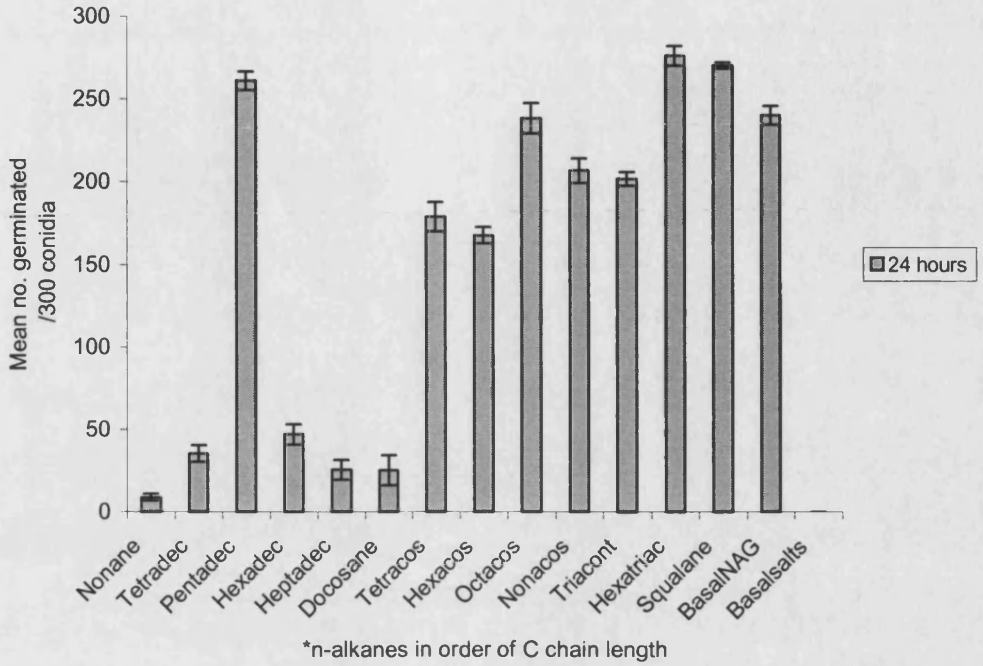
3.4.2 Germination on authentic non-polar lipids

A variety of non-polar lipids were tested for their potential ability to promote germination in *M.anisopliae* var. *acridum*. They were all hydrocarbons and, with the exception of nonane, have been found on the surface of the desert locust epicuticle, both in the present work (see section 3.3) and previously published work (Oraha and Lockey, 1990). The hydrocarbons were incorporated into basal salts agar as described previously (section 3.4.1). A positive control treatment of basal salts agar and *N*-acetyl glucosamine (*N*AG, a readily available source of carbon) was prepared, together with a negative control treatment of basal salts agar only.

The compounds are placed in ascending order of chain length in Fig. 3.29. The greatest germination occurred on plates incorporating those longer chain hydrocarbons. The highest level of germination was recorded on hexatriacontane (a 36 carbon *n*-alkane), followed by squalane (a branched alkane), pentadecane (a 15 carbon *n*-alkane) and octacosane (a 28 carbon *n*-alkane). These lipids all stimulated mean germination of the fungus of over 250 per 300 conidia. This was greater than the germination supported by basal salts agar containing polar lipids. The mean germination figures of the four most successful alkanes were also higher than the positive control treatment, a readily available monosaccharide (*N*AG).

Germination on most of the shorter chain hydrocarbons such as nonane (9 carbon atoms), tetradecane (14 carbon atoms), hexadecane (16 carbon atoms), heptadecane (17 carbon atoms) and docosane (22 carbon atoms) was lower than the positive control.

Figure 3.29: Germination of *M.anisopliae* var. *acridum* on authentic non-polar lipids (mean ± sd, n = 5)



* For a full list of *n*-alkanes see Table 2.3, Chapter 2

3.4.3 Germination on glucose and amino acids

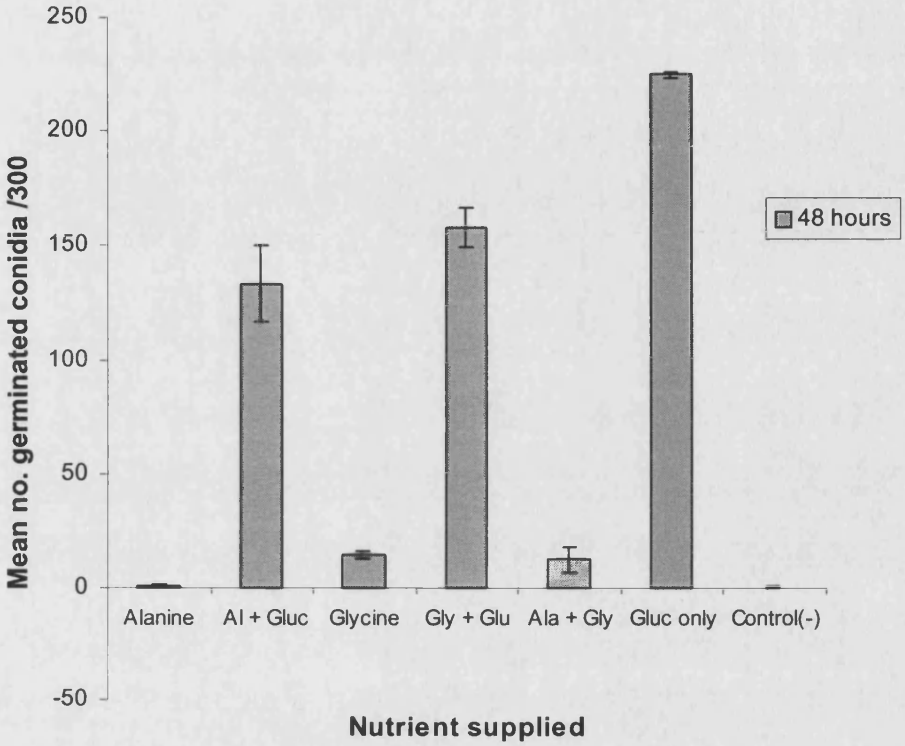
In section 3.3.0 of this chapter, the glucose and protein content of the pentane, chloroform and methanol extracts from desert locust hind wings was determined before and after fungal attack. Amino acid analysis was also performed on the methanol extract. The assays showed a reduction in the levels of all of these nutrients after fungal attack especially those soluble in methanol. Assays on control treatments (Table 3.6) established that the reduction was not due to an ageing effect of an excised hind wing, but rather fungal utilisation.

Experiments were carried out using authentic compounds to establish if glucose and or amino acids, at concentrations calculated to be present in the epicuticle, could

support germination. Alanine and glycine were selected for study as they were the most abundant amino acids present in the methanol extract

Figure 3.30 shows the results of this investigation. Germination occurred slowly on these nutrients. However, by 48 h 224.8 ± 1.5 per 300 conidia had germinated on 0.13mM glucose. Germination was less successful on amino acids, when supplied alone or in combination. Mean germination on 0.082 μmoles alanine was only 0.4 ± 0.2 per 300 conidia 48 h post inoculation. Germination on glycine was only a little better. When the amino acids were combined a mean germination of 12.4 ± 5.3 of 300 conidia was the result. Germination of the fungus increased dramatically, however, when amino acids were combined with glucose. Interestingly, germination in those combinations was less than in glucose alone.

Figure 3.30: Germination of *M.anisopliae* var. *acridum* on authentic amino acids and glucose in various treatment combinations (mean ± sd, n = 5)



3.4.4 Germination on pentane, chloroform and methanol solvent extracts from excised *S.gregaria* hind wings, before and after fungal attack

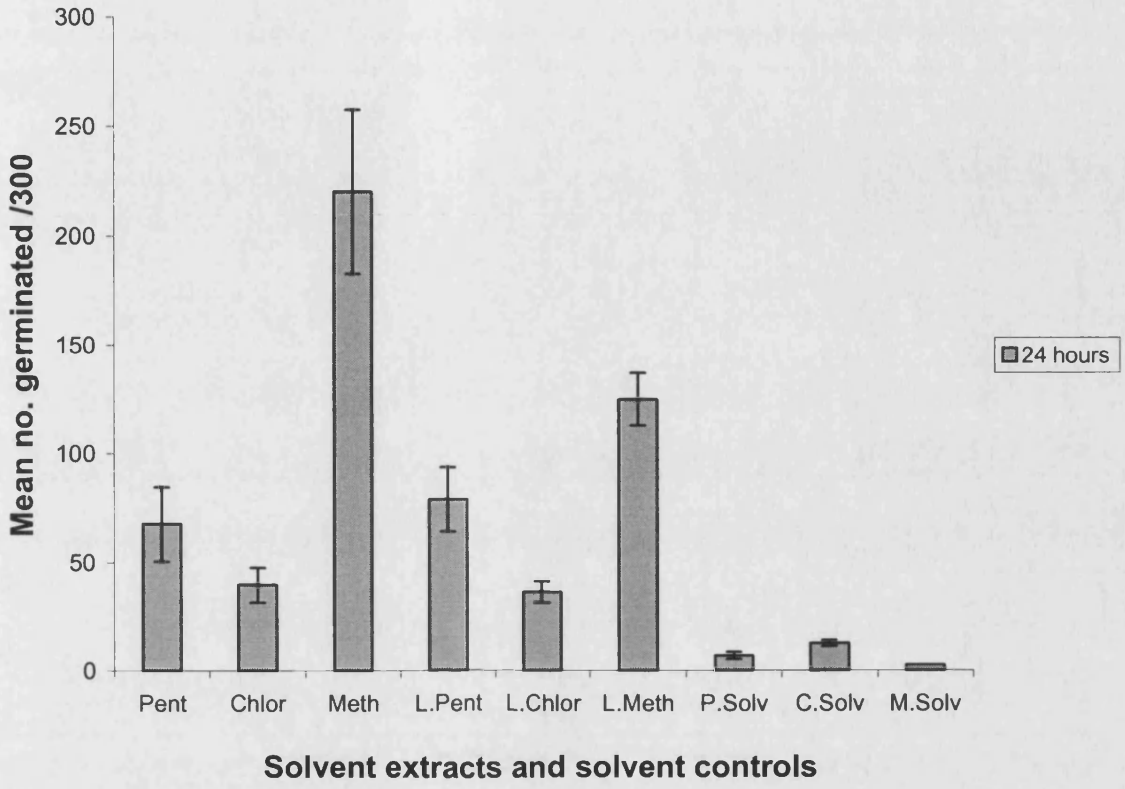
This experiment was designed to measure the ability of *M.anisopliae* var. *acridum* to germinate *in vitro* on the nutrients that were extracted by solvents from the surface of the desert locust hind wing before and after inoculation with fungus.

The results of the study are shown in Figure 3.31. Pentane, chloroform and methanol extracts from sterilised desert locust hind wings were placed on individual glass microscope slides, and, after the solvent had evaporated, inoculated and incubated. After 24 h the mean number of germinated conidia per 300 counted was counted.

Mean germination on the pentane extract was 67.6 ± 17 per 300 conidia, and on the chloroform extract 39.6 ± 8 of 300 conidia. These counts were considerably less than the 220.3 ± 37.4 per 300 conidia achieved on the methanol extract. This result highlights the importance of the compounds in the methanol extract and is consistent with the reduction in germination on wings that had been extracted with methanol (see section 3.1.6).

Extracts were also made from hind wings that had already been attacked once by *M.anisopliae* var. *acridum*, then fungus removed. The germination results show that nutrients remained after a 4 x 10^6 conidia ml^{-1} dose of fungus had been allowed to germinate on the hind wing surface. Germination on pentane and chloroform extracts post fungus treated was not different from that on control wings. However, the mean germination on the post fungus methanol extract, 125 ± 12.1, was reduced to almost half that on the original methanol extract. There was some germination on the solvent controls suggesting the presence of impurities, though re-distilled HPLC grade solvents were used.

Figure 3.31: Germination of *M.anisopliae* var. *acridum* on pentane, cloroform and methanol extracts of excised *S.gregaria* hind wings (mean ± sd, n = 5)



*L.Pentane, L.Chloroform and L.Methanol = previously treated with laminarinase to remove a previous 24 h inoculation with *M.anisopliae* var. *acridum*. P.solvent = pentane solvent only, C.solvent = chloroform solvent only and M.solvent = methanol solvent only

3.4.5 Germination on 'bands' separated by TLC of methanol extract from excised *S.gregaria* hind wings

As a previous experiment (3.4.4) had shown a very high germination of *M.anisopliae* var. *acridum* conidia on the methanol extract *in vitro*, analysis was carried out to determine further the composition of this extract. It was established, in sections 3.3.4 and 3.3.6 that amino acids and glucose are present. Initially, Thin Layer Chromatography (TLC) was employed.

Varying numbers of bands were recorded for the methanol extract, depending on the solvents and solvent ratios used to develop the chromatograms.

A 90:20:2 hexane: diethyl ether: acetic acid mixture of solvents separated the methanol extract into four bands, whereas a 80:20:2 mixture of the same solvents separated the methanol extract into a different set of four bands and a 50:50:2 mixture of solvents separated a further different set of 3 bands. None of these bands corresponded to authentic lipid and non-lipid standards run alongside. Therefore, band composition was determined, after derivatisation, by GC-MS (see section 3.3.3).

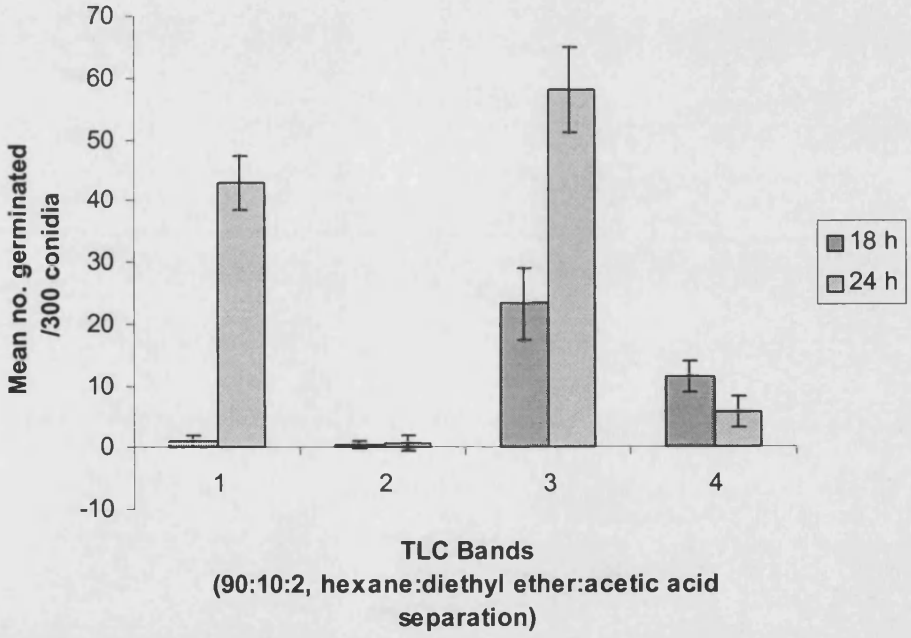
Figures 3.32, 3.33 and 3.34 show the results of the germination tests on extracts made from the TLC bands. Silica was removed from the bands, which had been scraped from the TLC plates, with PTFE Acrodisc© syringe filters, and then suspended in methanol solvent. This suspension was then transferred to glass microscope slides and the solvent allowed to partially evaporate. The slides were

then inoculated with *M.anisopliae* var. *acridum* conidia and germination was determined at 18 and 24h post-inoculation.

90:10:2 solvent system

Bands 1 and 3 gave the highest germination, though there was a delay with the extract from band 1.

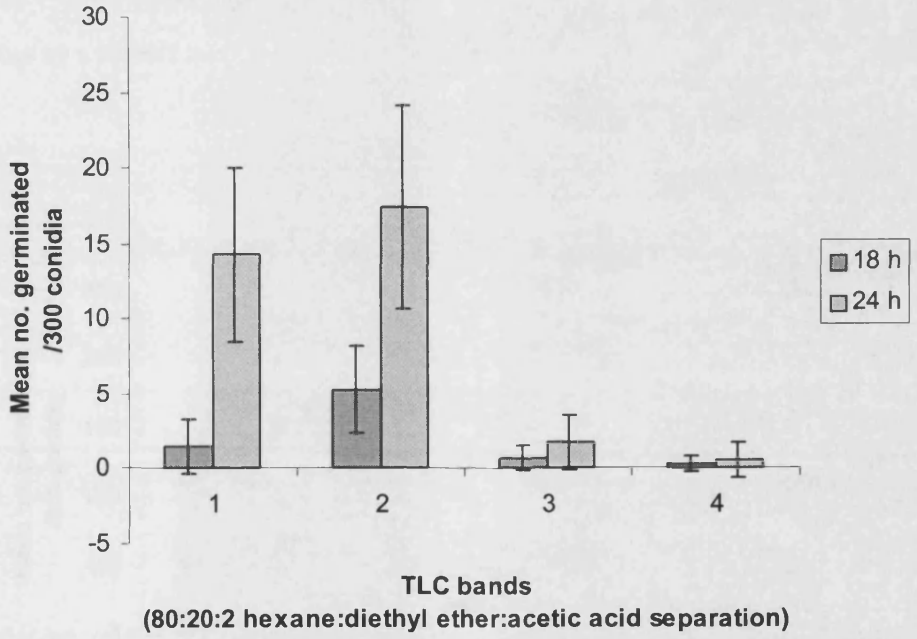
Figure 3.32: Germination of *M.anisopliae* var. *acridum* on bands separated from *S.gregaria* hind wings by a 90:10:2 hexane:diethyl ether:acetic acid solvent mix (mean ± sd, n = 7)



80:20:2 solvent system

Extracts from bands 1 and 2 consistently gave the highest germination, which was significantly greater than either band 3 or 4.

Figure 3.33: Germination of *M.anisopliae* var. *acridum* on bands separated from *S.gregaria* hind wings by a 80:20:2 hexane:diethyl ether:acetic acid solvent mix (mean ± sd, n = 7)

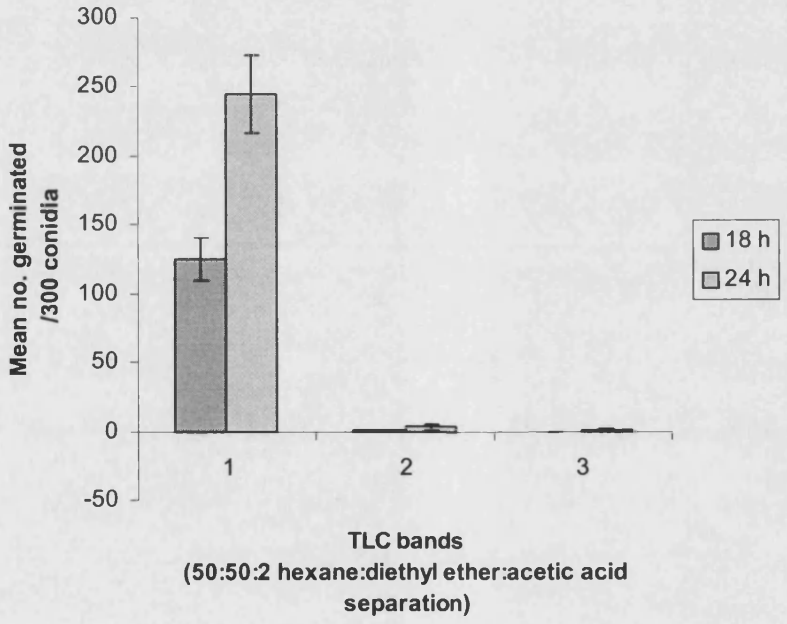


50:50:2 solvent system

Three bands were separated from the methanol extract with this solvent mix, but only the first band supported germination (125.28 ± 15.6 per 300 conidia at 18 h post inoculation). Indeed by 24 h post inoculation germination 245 ± 27.9 per 300 conidia was achieved, which is comparable to that recorded on whole, sterilised desert locust hind wings, of 268.6 ± 17.5 per 300 conidia.

Band 1 of this last TLC separation, as the band separated from the desert locust hind wing methanol extract that stimulated the greatest mean germination of all the bands tested, was selected for identification by GC-MS. The purified band was re-suspended in methanol and derivatised with Methyl-8 concentrate, before injection into the GC-MS apparatus and identified as a single compound, a free fatty acid, 9,12-octadecenoic acid (linoleic acid).

Figure 3.34: Germination of *M.anisopliae* var. *acridum* on bands separated from *S.gregaria* hind wings by a 50:50:2 hexane:diethyl ether:acetic acid solvent mix (mean ± sd, n = 7)



3.5.0 *S.gregaria* hind wing and *M.anisopliae* var. *acridum* surface structure

3.5.1 Cryo-SEM studies of excised *S.gregaria* hind wings and *M.anisopliae* var. *acridum*

The following plates show cryo-scanning electron microscopy studies on detached *S.gregaria* hind wings subjected to various treatments, listed below:

Plate One: Standard preparation – sterilised hind wings

Figure 3.35

This cryo-scanning electron micrograph (Cryo-SEM) shows a desert locust *(S.gregaria)* hind wing at x 90 magnification. The specimen had been prepared using the standard procedure for all experiments using hind wings in this research, through sterilisation with propylene oxide. Strengthening wing ribs are visible on this Cryo-SEM, as well as the smooth wing membrane between the ribs.

Figure 3.36

Figure 3.36 shows a higher magnification (x 5,500) Cryo-SEM of the standard, sterilised preparation of *S.gregaria* hind wing. At higher magnification the 'smooth' surface that appears at lower magnification is shown to be covered with wax-like and other small particles of 1 μm and less in size.

Plate Two: Unsterilised hind wings

Figure 3.37

This Cryo-SEM shows a non-sterilised *S.gregaria* hind wing preparation at a low magnification of x 500. The same smooth surface observed in sterilised *S.gregaria*

hind wings is evident in this preparation, which shows the area between wing ribs, suggesting that the sterilisation procedure has no qualitative effect on the hind wings.

Figure 3.38

Figure 3.38 is a higher magnification Cryo-SEM of the non-sterilised hind wing preparation (x 1000). This micrograph shows a 'wing spine' that can be visible to various degrees on all preparations of hind wings present in this study, depending on the amount of cuticular lipid has been distributed on the surface of the wing. As the magnification of this specimen was not a great as for the sterilised wing praparation, the same wax granules or other small particles of $\geq$ 1 μm are not visible in this micrograph.

Plate Three: Fungal infected hind wing

Figure 3.39

This Cryo-SEM shows a *S.gregaria* hind wing that has been inoculated with *M.anisopliae* var. *acridum* (concentration) spore suspension for 24 h. Germinating conidia are shown on a hind wing at a magnification of x 45. Conidia have germinated a droplet of conidia suspension applied to the hind wing using the Potter tower apparatus (described in section 2.2.1,Chapter 2).

Figure 3.40

This Cryo-SEM shows an infected *S.gregaria* hind wing at higher magnification (x 4,300). A single germling is featured, and the germ tube, appressorium and mucilagenous adhesive that promotes conidial attachment are clearly visible. The β

1, 4 glucans in this mucilage are hydrolysed by the laminarinase to bring about removal of these germlings (shown in figure 3.41 and 3.42)

Plate Four: Laminarinase treated hind wings – post-fungal infection

Figure 3.41

Figure 3.41 shows a *S.gregaria* hind wing preparation that has been inoculated with *M.anisopliae* var. *acridum* for 24 h, which has then been treated with a solution of laminarinase enzyme to facilitate removal of the germlings. 'Clearing zones' are shown on this Cryo-SEM in areas where the germlings had grown. Material seems to have been removed from the hind wing in these areas. This may correspond to surface lipids

Figure 3.42

Figure 3.42 shows the same laminarinase-treated preparation at a greater magnification (x 3,300). The absence of surface particles (shown in the standard hind wing preparation of figure 3.36) from areas where *M.anisopliae* var. *acridum* germlings had been removed, is clearly evident at this magnification. The following Cryo-SEM (figure 3.43) shows that the 'clearing zones observed in this preparation are due to the fungal infection rather than to treatment of the hind wings with laminarinase enzyme (see figure 3.43, overleaf).

Plate Five: Laminarinase treated hind wings – no fungal infection

Figure 3.43

Figure 3.43 shows a hind wing preparation at a magnification of x 50. This preparation has been treated with the same laminarinase enzyme solution as the hind wing shown in Figures 3.41 and 3.42, but without being inoculated with *M.anisopliae* var. *acridum*. The appearance of the hind wing is similar to the standard hind wing preparation, shown in Figure 3.36. This suggests that the laminarinase enzyme solution does not change the qualitative appearance of the hind wing, but simply allows for removal of *M.anisopliae* var. *acridum* germlings in attacking the mucilagenous adhesive used by the conidia to adhere to the hind wing surface.

Figure 3.43(a) shows a higher magnification image (x 200) of a laminarinase treated hind wing that had not been infected with fungus.

Plate Six: Solvent-treated wings –

Figure 3.44

This Cryo-SEM details a hind wing preparation which has been treated with pentane, chloroform and methanol solvents in order to remove all polar and non-polar cuticular lipids from the hind wing epicuticular surface (x 50 mag). The wing spines (shown by arrows) are more clearly visible than in other treatments that have not been solvent treated (*e.g.* Figures 3.35 and 3.37) due to the removal of cuticular lipids that can cover these spines or collect around them as a result of grooming.

Figure 3.45

Figure 3.45 shows a higher magnification image (x 500 mag.) of a solvent treated *S.gregaria* hind wing. The arrows marked on the Cryo-SEM show the alteration caused to the structure of the hind wing as a result of solvent treatment and/or mechanical forces during the refluxing procedure. This emphasises the importance of using hind wings as a source of cuticular lipid, as opposed to whole insects. Any damage to the cuticle of whole insects during extraction would allow lipid contaminants from within the insect (*e.g.* fat body and haemolymph lipids) to enter the extractant. The hind wing of an insect consists of a bi-layer of cuticle only, so damage to this cuticle during extraction yields no contaminants.

Plate Seven: Axenic-reared hind wings

Figure 3.46 and 3.47

Figures 3.46 and 3.47 (low and high magnification images respectively) show Cryo-SEMs of a hind wing excised from a desert locust which had been reared under axenic conditions. Both figures show clearly visible wing spines. When compared to the standard sterilised hind wing preparation (Figure 3.35), it appears that there are less surface particles present. The presence of particles on the surface of the standard sterilised preparation could be in some part due to the activity (metabolic and mechanical) of micro-organisms. As hind wings from locusts reared under axenic conditions are not exposed to micro-organisms, the number of particles present on the surface is less.

Plate Eight: Hind wings from newly moulted insects

Figure 3.48

This Cryo-SEM shows a low magnification image (x 110) of a *S.gregaria* hind wing preparation from a newly moulted adult. As with hind wing preparations from axenic-reared and extracted hind wing preparations, the wing spines are highly visible, suggesting little cuticular lipid is present at this stage of development.

Figure 3.49

When compared to a high magnification standard preparation of *S.gregaria* (Figure 3.36 at 5,500mag) it is evident that the highly magnified image (x 1,700) of a newly moulted desert locust hind wing has a great deal less surface material present on the cuticle. This is likely to be due to the absence of cuticular lipids and other particles from the cuticular surface. However, the magnifications of these images are not exactly matched (x 5,500 *vs.* x 1,700)

3.5.2 Atomic Force Microscopy studies on excised *S.gregaria* hind wings

The following plates detail an atomic force microscopy study on excised *S gregaria* hind wings after various different treatments:

Plate Nine: standard sterilised hind wing preparation

Figures 3.50 and 3.51

This Atomic Force Micrograph (AFM) image shows the topography of the surface of a sterilised desert locust hind wings (standard preparation) at very high resolution. The surface of the wing appears smooth and undulating with folds and grooves which may provide conditions that create humid microclimates on the wing. As

Figure 3.40 (Plate Three) shows, the average size of a *M.anisopliae* var. *acridum* conidium is ~ 6 μm in length. The crevice shown on the atomic force micrograph, Figure 3.51, would perhaps provide a pocket of high humidity on the hind wing where conidial germination could take place. The cuticular lipids and other chemicals creating and lining the depression, would provide the nutrients required for fungal growth.

Plate Ten: non-sterilised hind wing preparation

Figure 3.52 and 3.53

This AFM shows the topography and surface structure at the μm level of a *S.gregaria* hind wing that had not been treated with propylene oxide for sterilisation. Figures 3.52 and 3.53 show similar images to the sterilised preparation shown in Figures 3.50 and 3.51, in that the topography is smooth but undulated to form crevices and ridges, which could provide suitable points of adhesion for conidia.

Plate Eleven: post-fungal infection + laminarinase treated hind wing preparation

Figures 3.54 and 3.55

These AFM images are remarkably different to those shown in Figures 3.50-3.53. The hind wings in this preparation had been inoculated with *M.anisopliae* var. *acridum* for 24 hours before removal of the germlings with a laminarinase enzyme solution. The landscape depicted in these images appears uneven when compared to the smooth undulations on Figures 3.50-3.53 shown previously.

Although Figure 3.54 represents a larger area of the hind wing than Figures 3.50-3.53 by 10μm the surface appears jagged and rough in texture and does not resemble the even face of the standard and non-sterilised hind wing preparations.

Figure 3.55 shows an image of similar magnification to those shown in Figures 3.50-3.53, and the irregular appearance is still evident. Figure 3.56 overleaf shows a *S.gregaria* hind wing that has been treated with laminarinase, but that has not been inoculated with *M.anisopliae* var. *acridum*. The surface structure of the laminarinase-only (no fungus) preparation (Figure 3.56) corresponds to the images produced by the Atomic Force Microscopy of sterilised and non-sterilised hind wings. Therefore it can be seen that the rough surface shown in Figures 3.54 and 3.55 is due to the effect of *M.anisopliae* var. *acridum* on the cuticle's surface components during fungal growth on the wing epicuticle.

Plate Twelve: hind wings from *S.gregaria:* (i) uninfected but treated with laminarinase and (ii) reared under axenic conditions.

(i) Figure 3.56

As discussed in reference to Figures 3.54 and 3.55, Figure 3.56 shows an AFM image of a *S.gregaria* hind wing treated with laminarinase only, and which had not been inoculated with *M.anisopliae* var. *acridum*. The smooth, undulating surface shown in Figure 3.56 suggests that the laminarinase treatment does not cause the rugged topography of the hind wing observed in Figures 3.54 and 3.55, and that the only other variable present, the fungus, most probably caused the effect.

(ii) Figure 3.57

This AFM image shows a high-resolution view of a *S.gregaria* hind wing that has been excised from an animal reared under axenic conditions. As with AFMs produced for sterilised, non-sterilised and laminarinase-only hind wing treatments, the surface is even with occasional smooth, raised areas, suggesting that rearing insects under axenic conditions has no effect on the surface landscape of the hind wing when observed at this level of magnification.

PLATE ONE

Figure 3.35: Standard hind wing preparation: sterilised in propylene oxide x 90 mag.

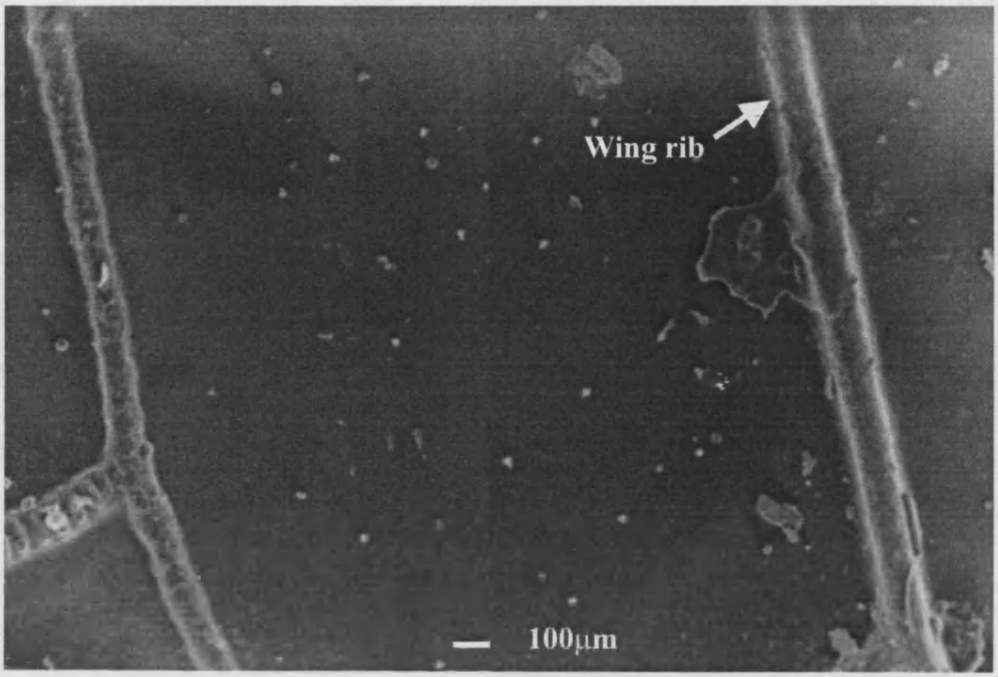


Figure 3.36: Standard hind wing preparation: sterilised x 5,500 mag.

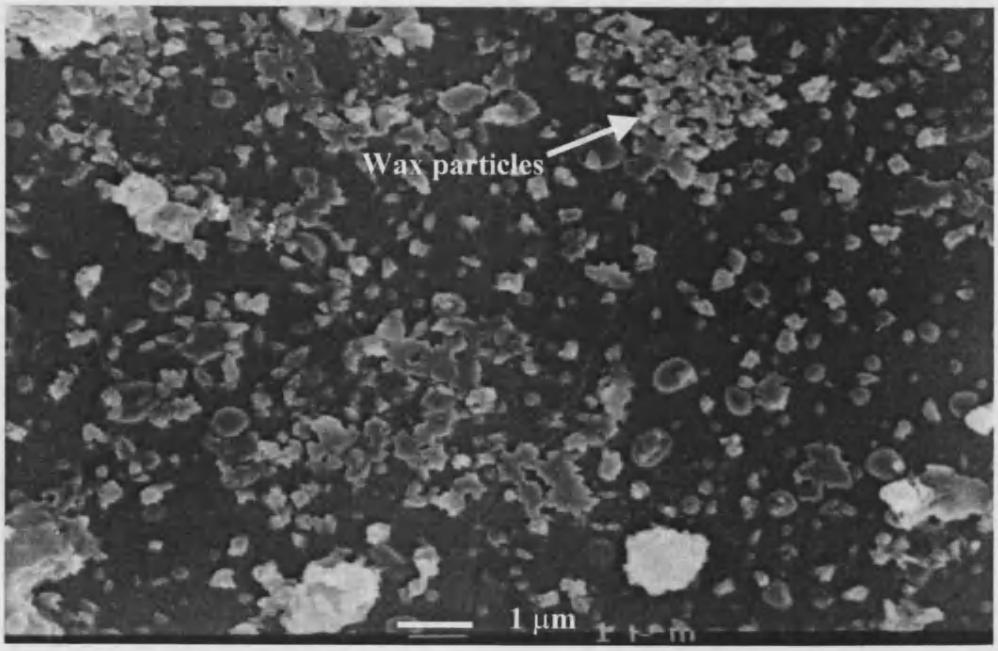


PLATE TWO

Figure 3.37: Unsterilised hind wing preparation x 500 mag.

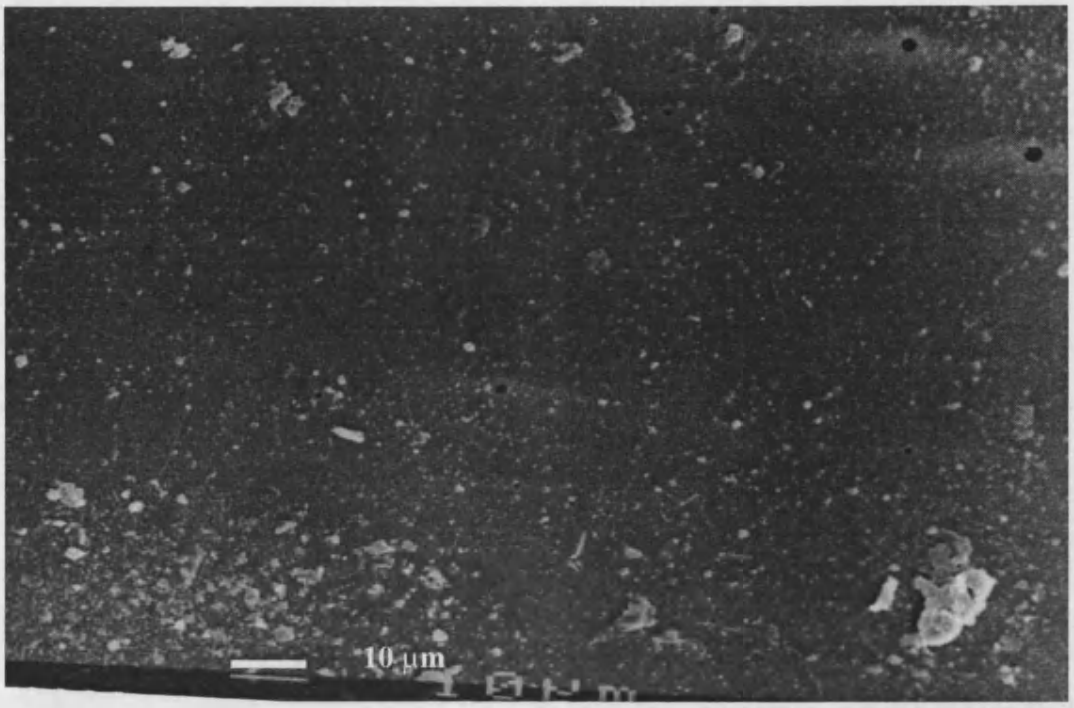


Figure 3.38: Unsterilised hind wing preparation x 1,000 mag

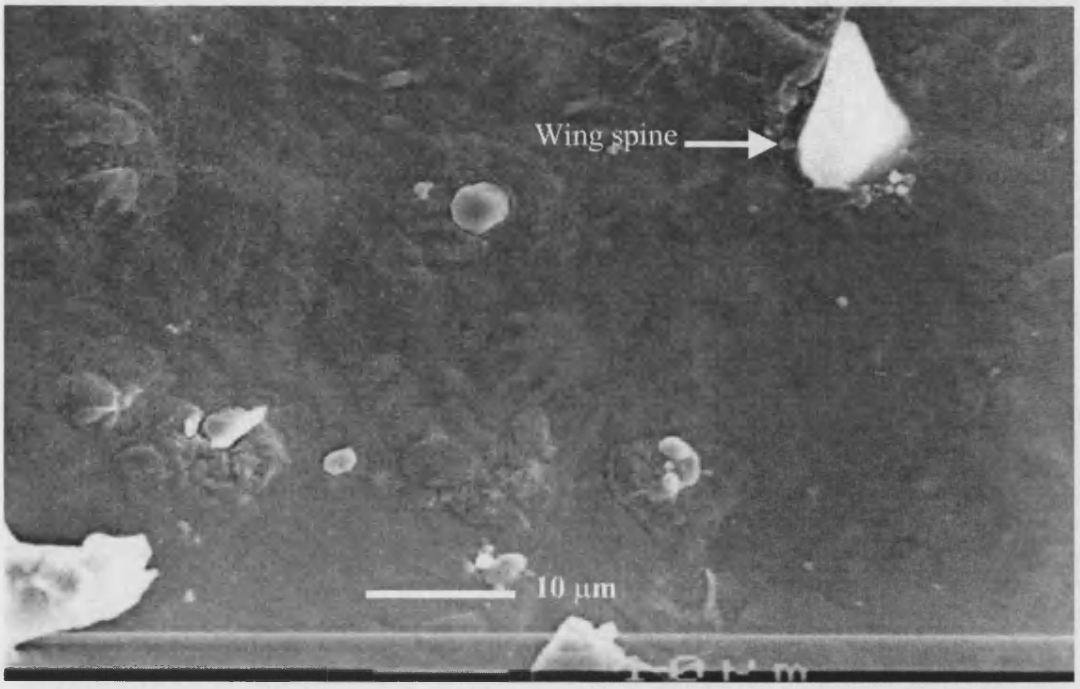


PLATE THREE

Figure 3.39: Fungal-infected hind wing preparation x 45 mag.

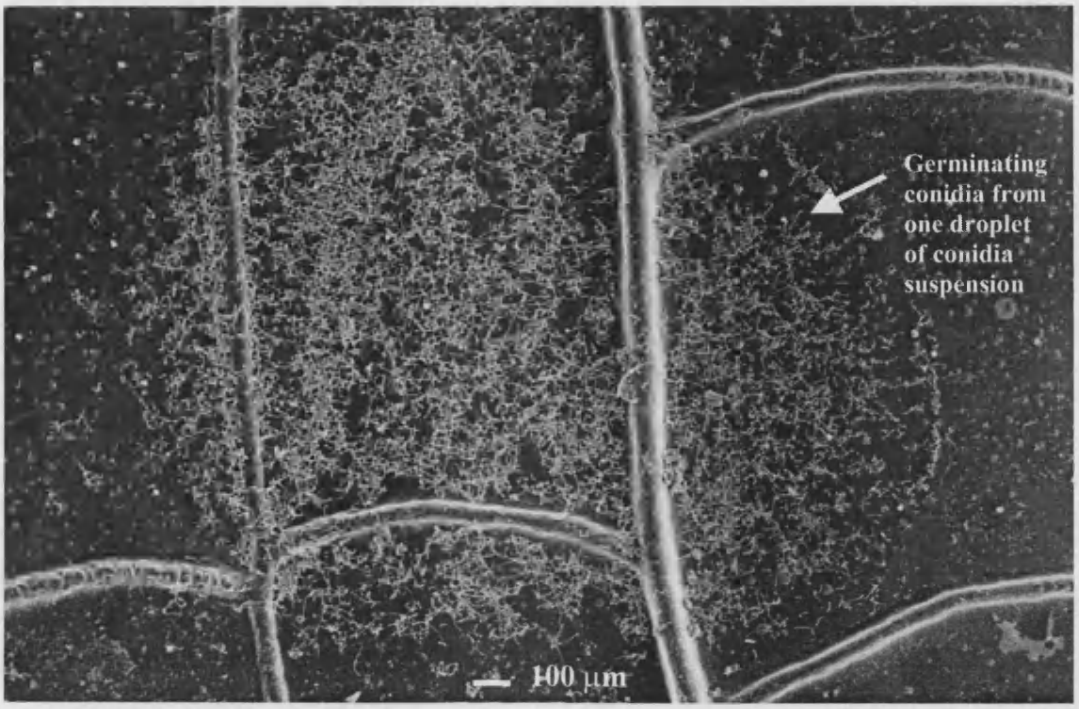


Figure 3.40: Fungal infected hind wing preparation x 4,300 mag.

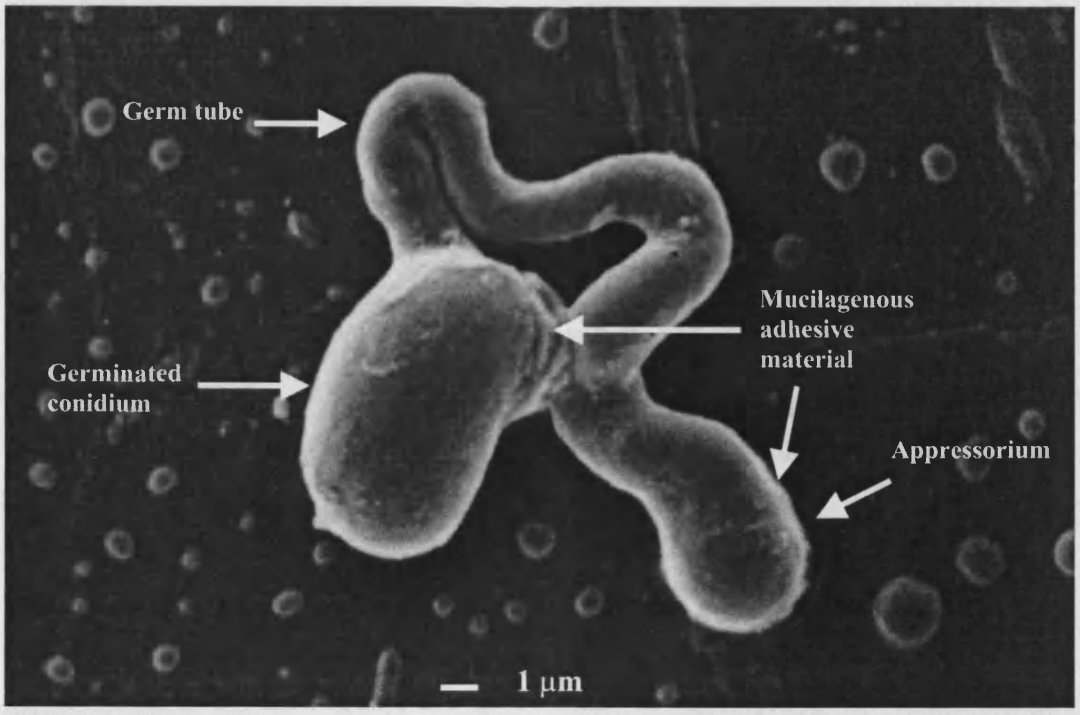


PLATE FOUR

Figure 3.41: Laminarinase treated infected hind wing preparation x 3,300 mag.

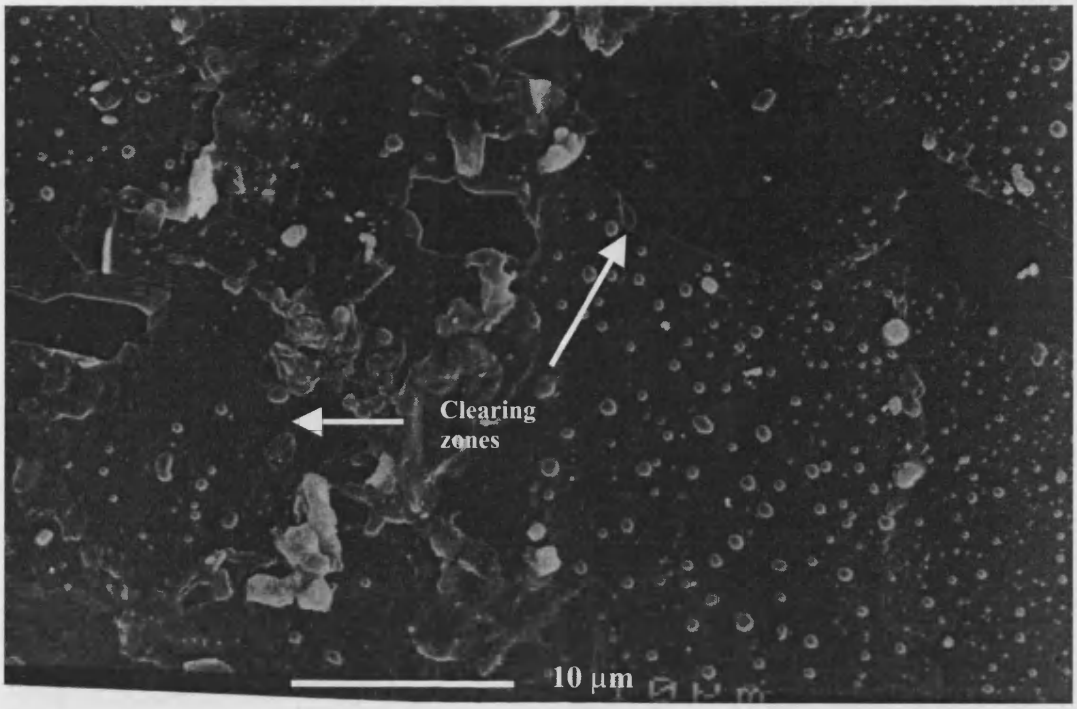


Figure 3.42: Laminarinase treated infected hind wing preparation x 1,900 mag.

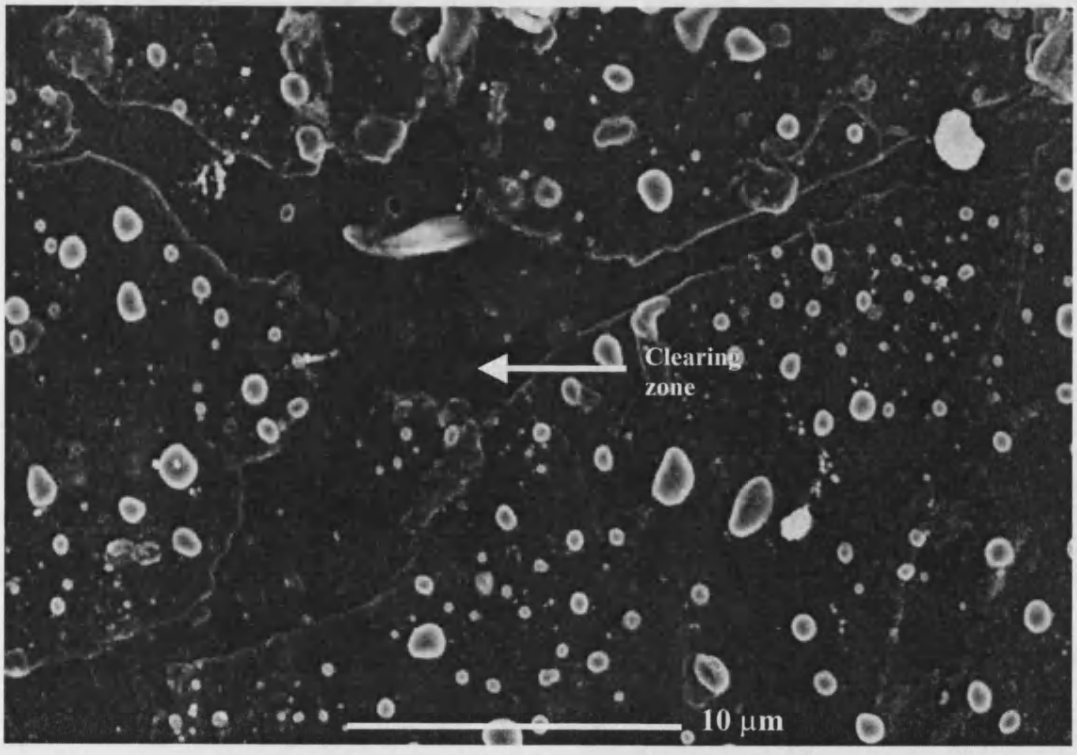


PLATE FIVE

Figure 3.43: Laminarinase treated uninfected hind wing preparation x 50 mag.

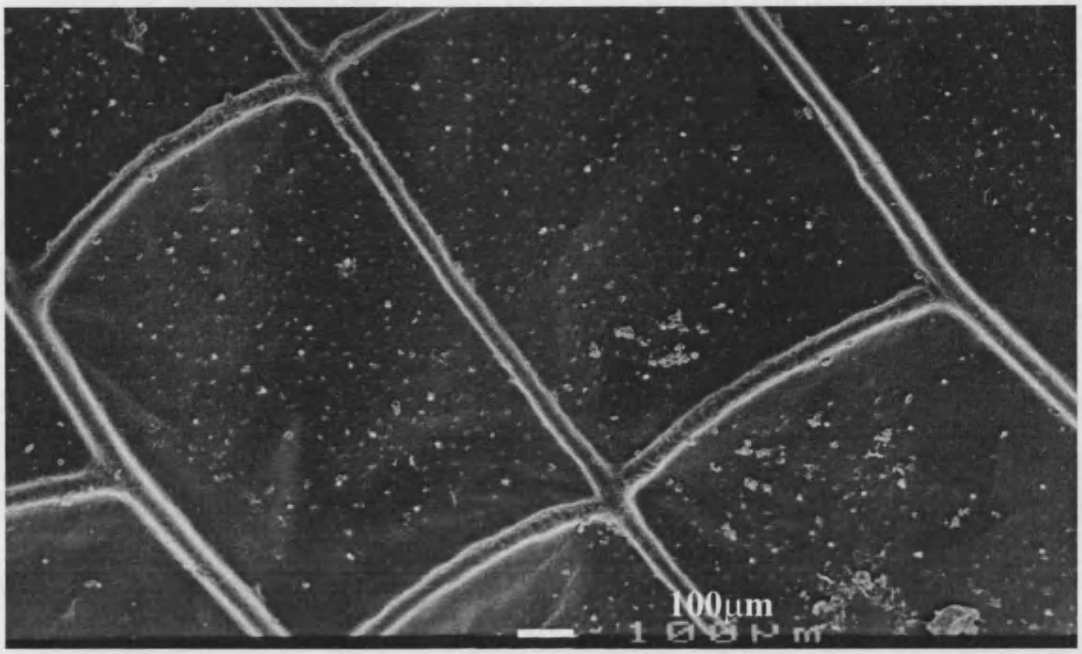


Figure 3.43a: Laminarinase treated uninfected hind wing preparation x 200 mag

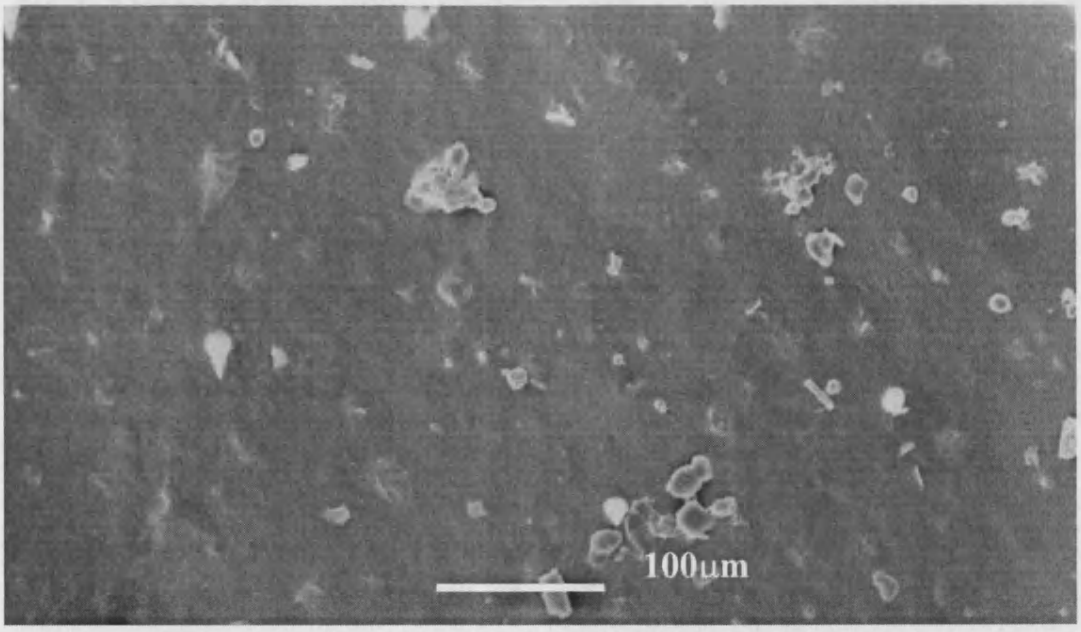


PLATE SIX

Figure 3.44: Solvent-treated hind wing preparation x 50 mag.

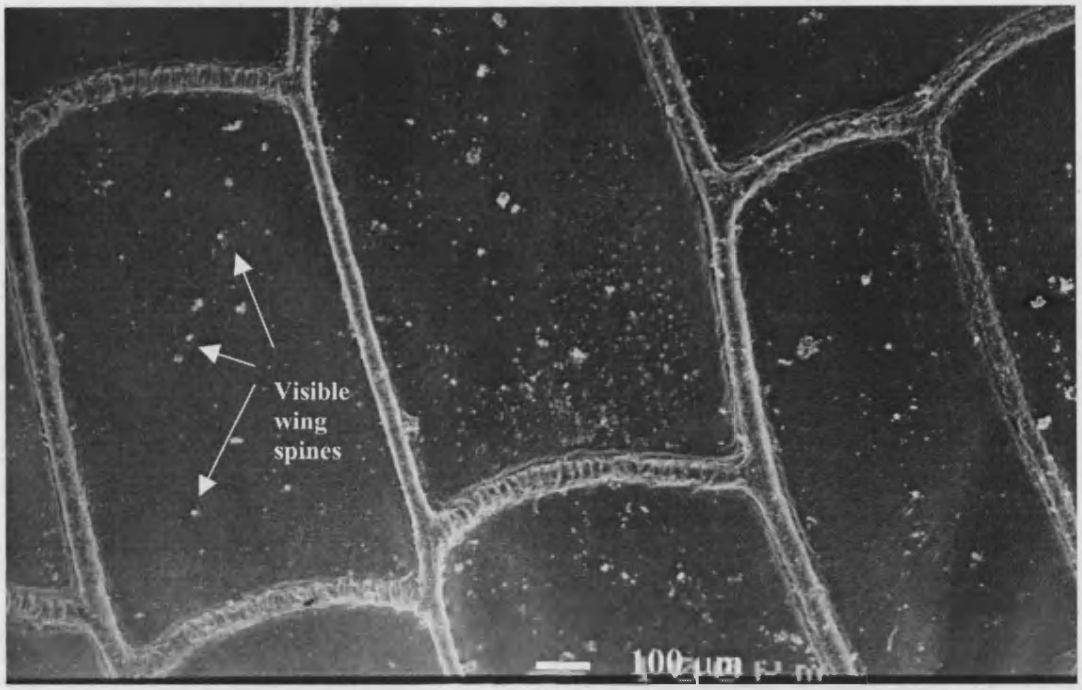


Figure 3.45: Solvent-extracted hind wing preparation: x 500 mag.

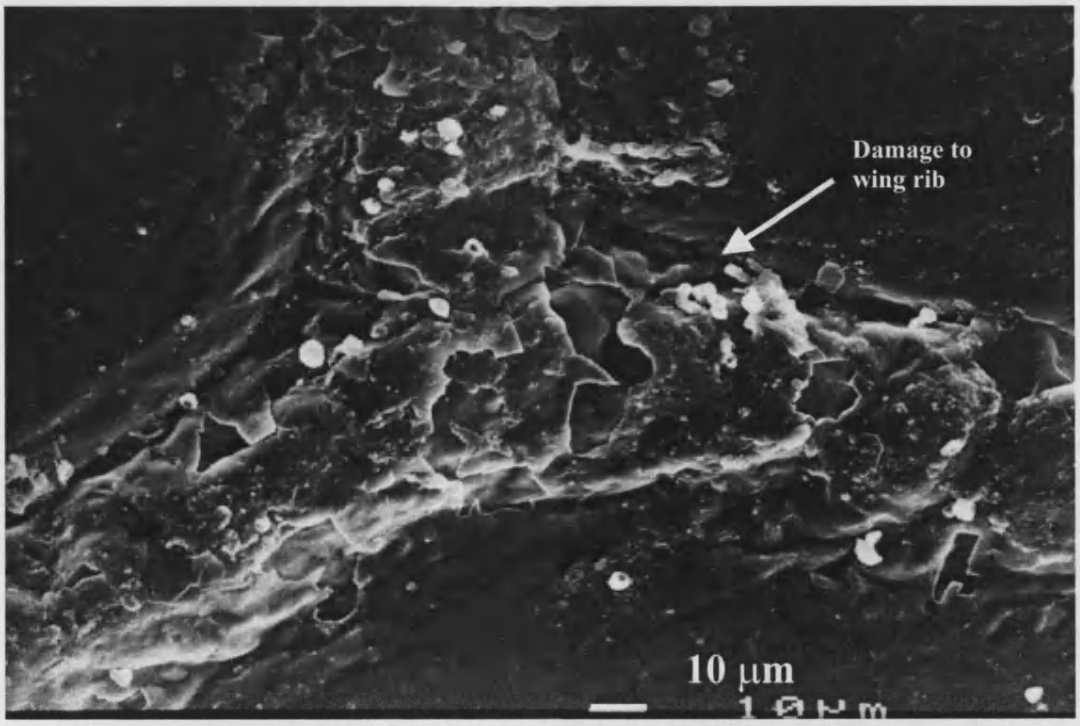


PLATE SEVEN

Figure 3.46: Axenic hind wing preparation x 120 mag.

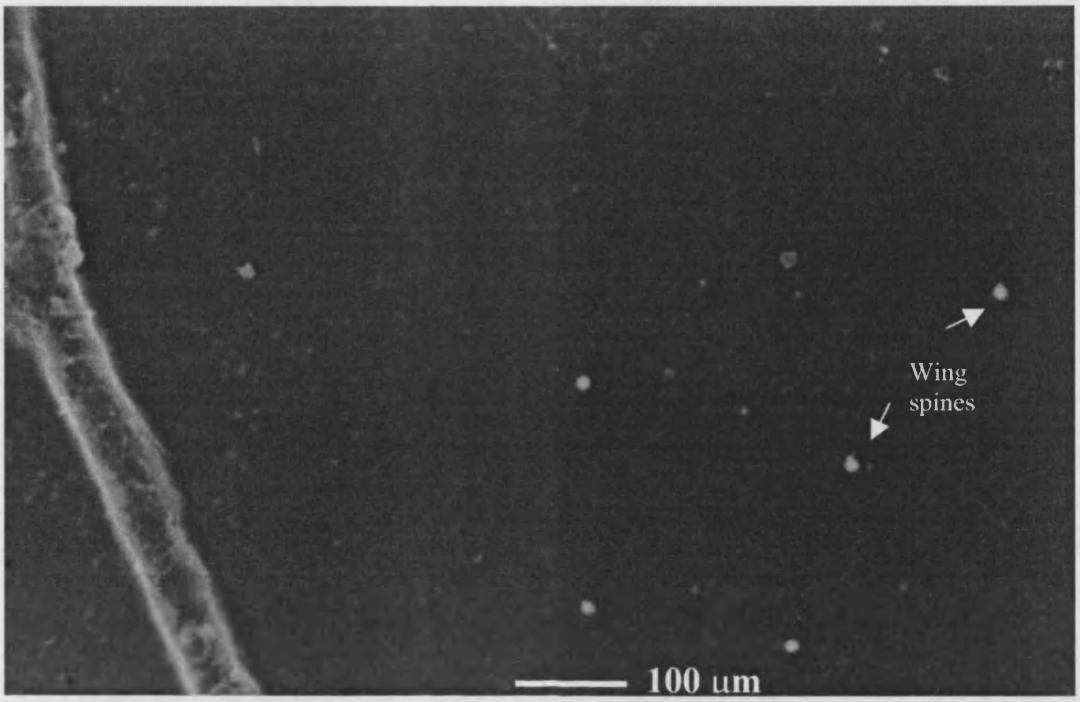


Figure 3.47: Axenic hind wing preparation: x 200 mag.

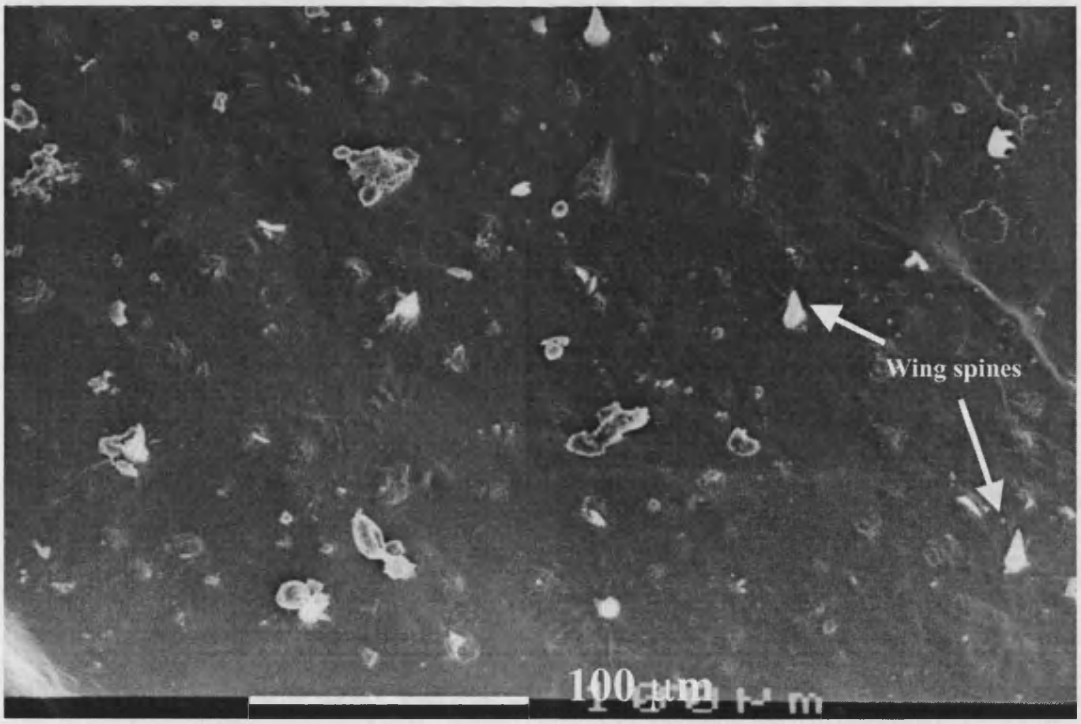


PLATE EIGHT

Figure 3.48: Newly moulted hind wing preparation x 110 mag.

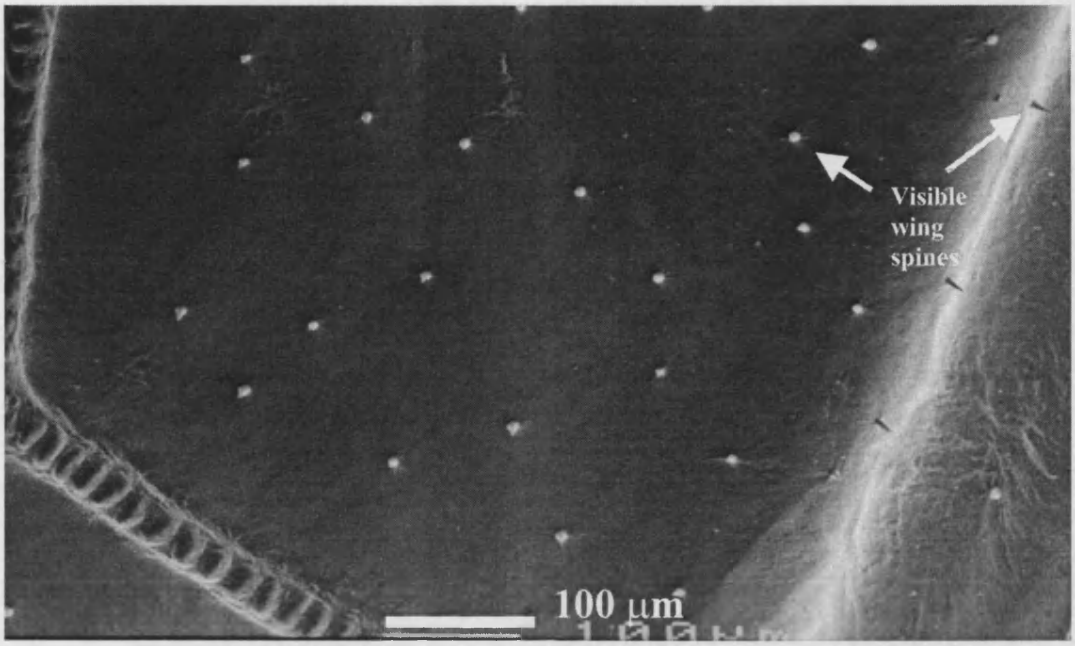


Figure 3. 49: Newly moulted hind wing preparation x 1,700 mag.

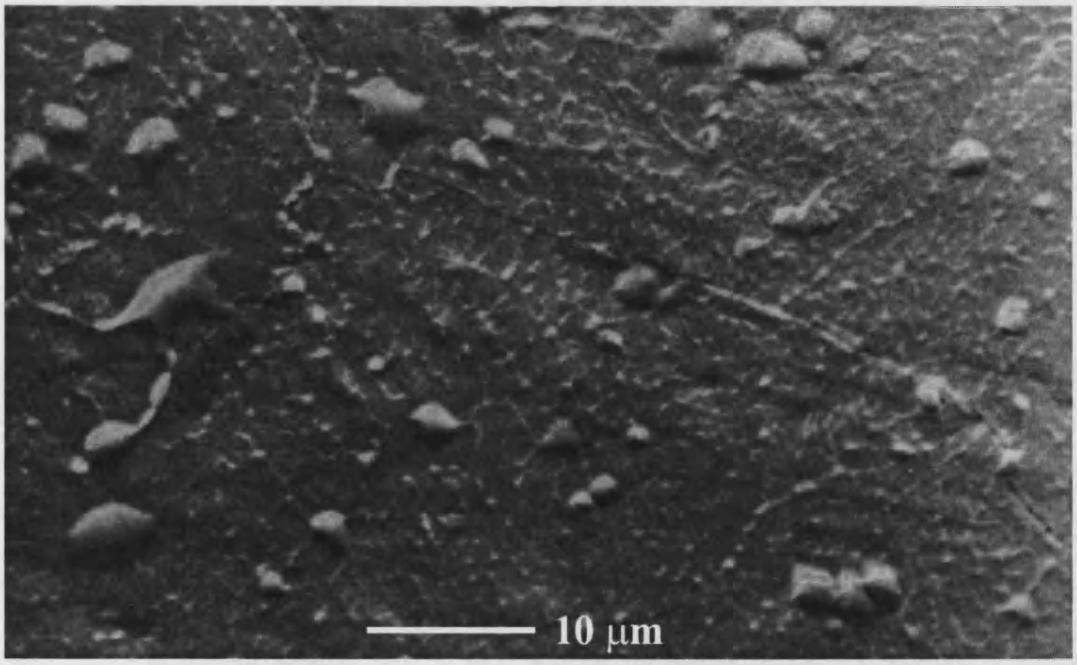


PLATE NINE

Figure 3.50: Atomic Force Micrograph of sterilised hind wing of desert locust

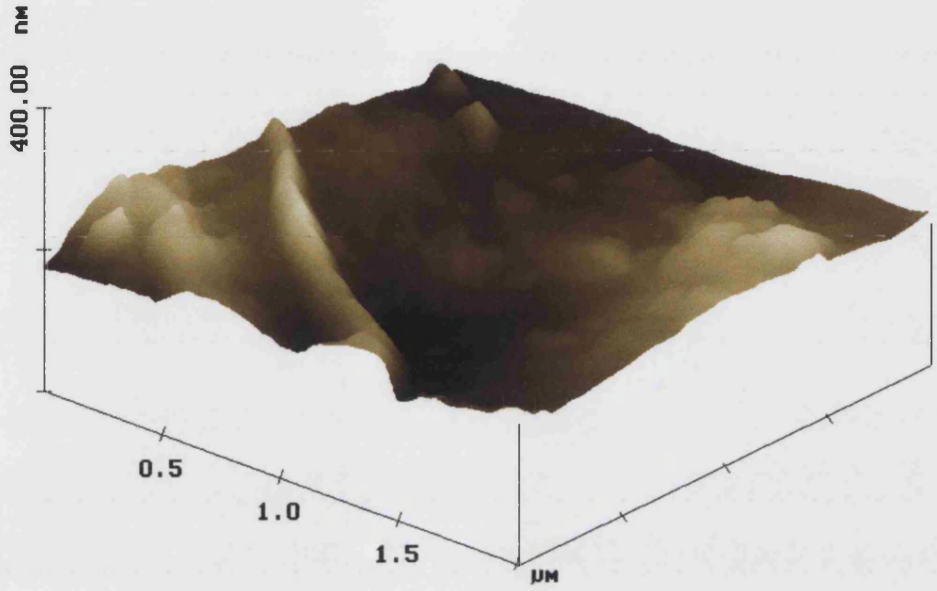


Figure 3.51: Sterilised hind wing of desert locust

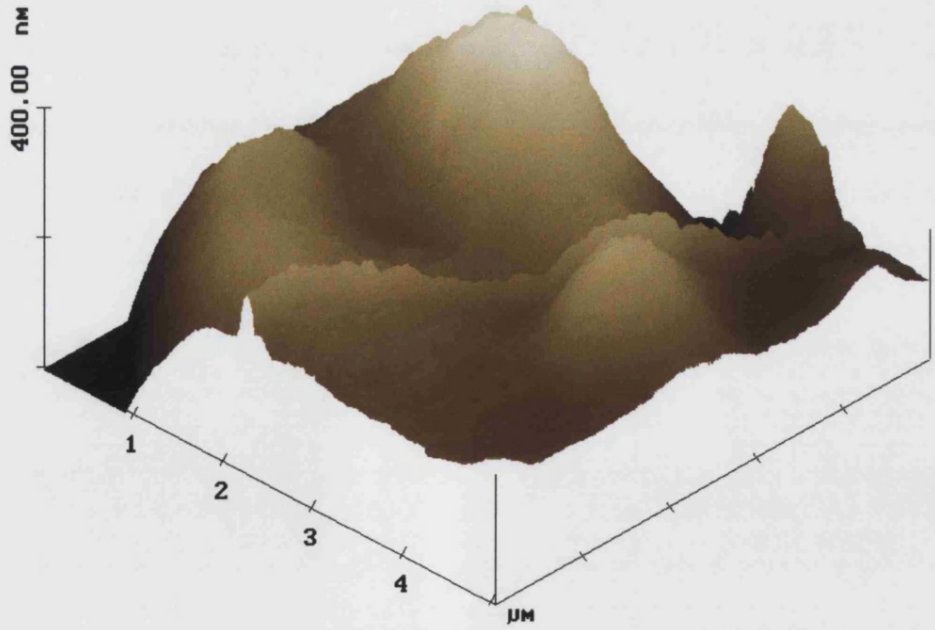


PLATE TEN

Figure 3.52: Atomic Force Micrograph of non-sterilised desert locust hind wing

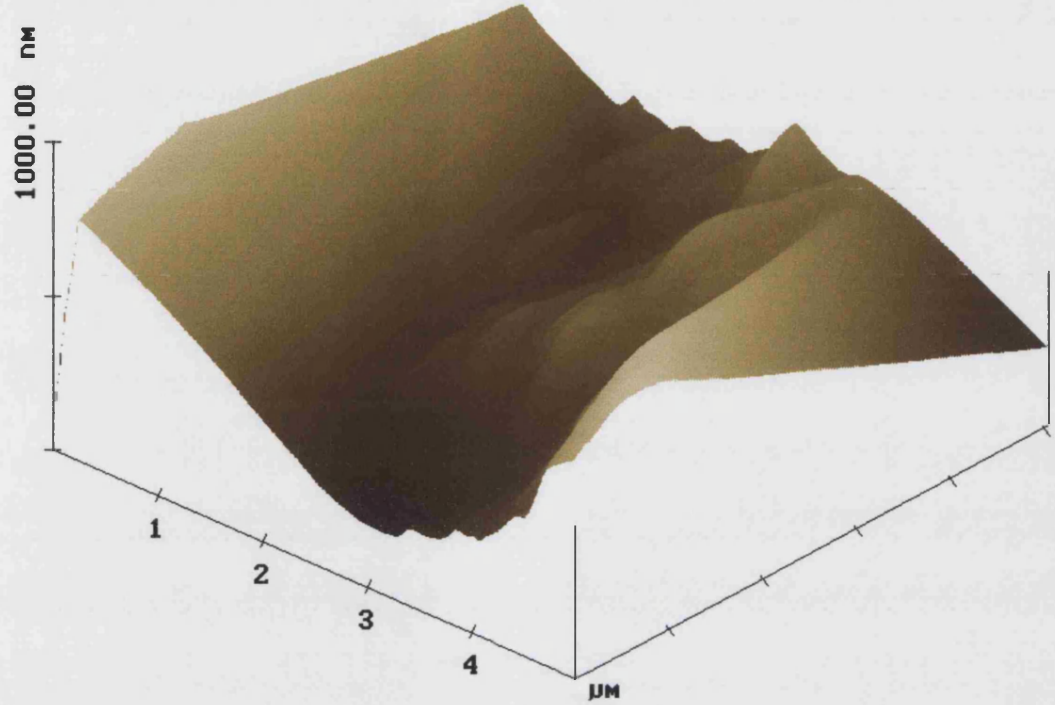


Figure 3.53: Non-sterilised hind wing of desert locust

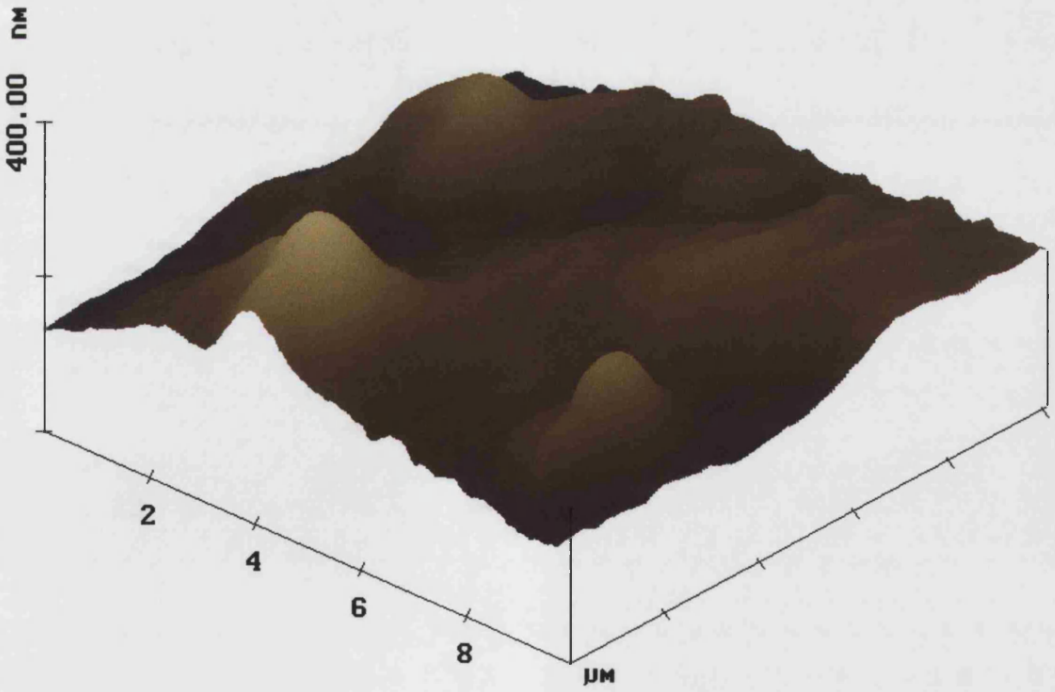


PLATE ELEVEN

Figure 3.54: Atomic Force Micrograph of desert locust hind wing post-fungal infection

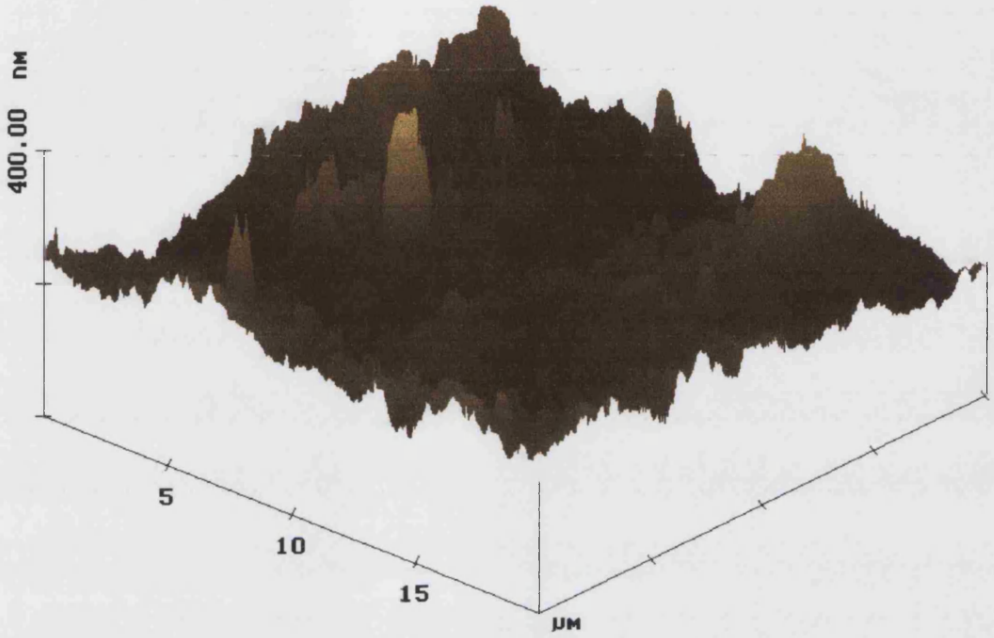


Figure 3.55: Desert locust hind wing post-infection

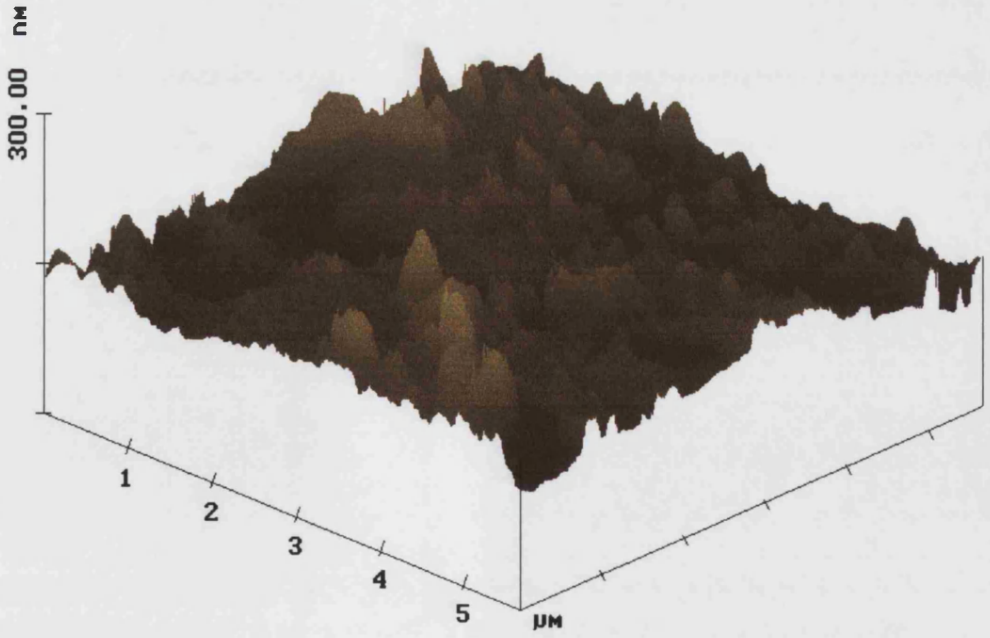


PLATE TWELVE

Figure 3.56: Atomic Force Micrograph of desert locust hind wing uninfected but treated with laminarinase

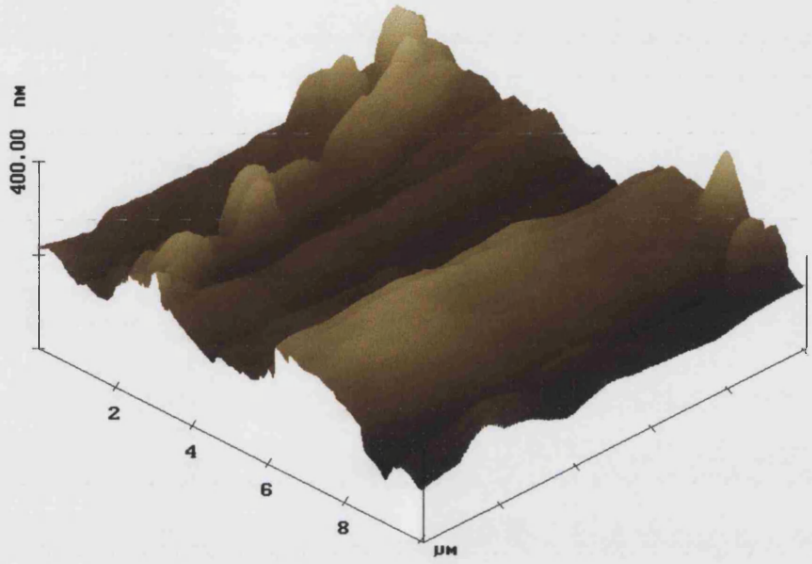
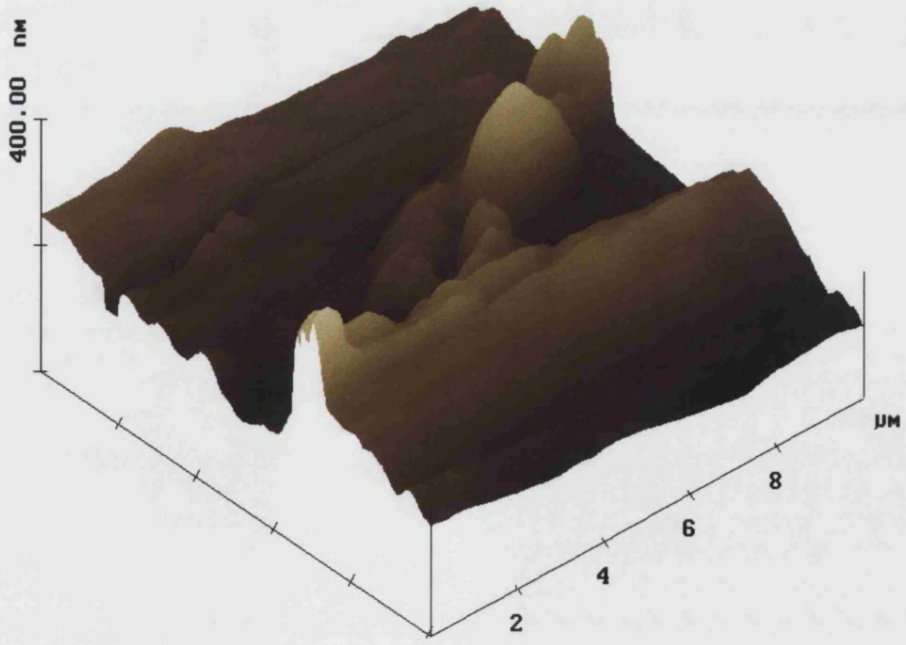


Figure 3.57: Hind wing from desert locust reared under axenic conditions



ix Appendix I

Media and buffer recipes:

1) Sabouraud's Dextrose Agar (SDA)

	Full strength ($g\ l^{-1}$)	¼ strength ($g\ l^{-1}$)
Mycological peptone	10	2
Glucose	40	10
Agar	20	20
Yeast extract	20	5

2) 2% Water Agar

	$g\ l^{-1}$
Agar	20

3) Cooper and Woods Basal Salts

Stock solution ($g\ l^{-1}$)		Trace solution ($g\ l^{-1}$)	
NaNO3	20	FeSO4.7H2O	0.02
4H2PO4	10	ZnSO4.7H2O	0.1
MgSO4.7H2O	5	CuSO4.5H2O	0.002
		MnCl2.4H2O	0.002
		NaMoO4.2H2O	0.002

4) McIlvaine's Buffer

Citric acid – 0.1M

di sodium hydrogen orthophosphate – 0.2M

Add 48.50ml 0.1M citric acid to 51.50ml *di* sodium hydrogen orthophosphate to make an enzyme buffer of pH 5.0.

x Appendix II

Statistical Analysis

Taken from Fowler, J. and Cohen, L., (1990) Practical Statistics for Field Biology, Wiley and Sons

1) Mann-Whitney U Test – example

e.g. To compare the germination of *M.anisopliae* var. *acridum* on sterilised wings *vs.* ¼ SDA

1. 242, 265, 268, 280, 298 n=5
2. 2, 6, 7, 8, 10 n=5

$$U_1 = n_1 n_2 + \frac{n_2 (n_2+1)}{2} - R_2$$

$$= 5 \times 5 + \frac{5(5+1)}{2} - 40$$

$U_1 = 0$

$$U_2 = n_1 n_2 + \frac{n_1 (n_1+1)}{2} - R_1$$

$$= 5 \times 5 + \frac{5(5+1)}{2} - 15$$

$U_2 = 25$

2) **Kruskal-Wallis Test – example**

e.g. To compare the germination at 12 h of *M.anisopliae* var. *acridum* on non-sterilised, sterilised and axenic hind wings

Non-sterilised	**Sterilised**	**Axenic**
298 (15)	288 (14)	256 (3)
246 (2)	242 (1)	282 (13)
279 (11)	280 (12)	266 (8)
269 (10)	268 (9)	261 (6)
260 (5)	265 (7)	257 (4)
n = 5	**n** = 5	**n** = 5
R = 43	**R** = 43	**R** = 34
$\mathbf{R^2}$ = 1849	$\mathbf{R^2}$ = 1849	$\mathbf{R^2}$ = 1156
$\mathbf{R^2/n}$ = 369.8	$\mathbf{R^2/n}$ = 369.8	$\mathbf{R^2/n}$ = 231.2

Σ $\mathbf{(R^2/n)}$ = 970.8

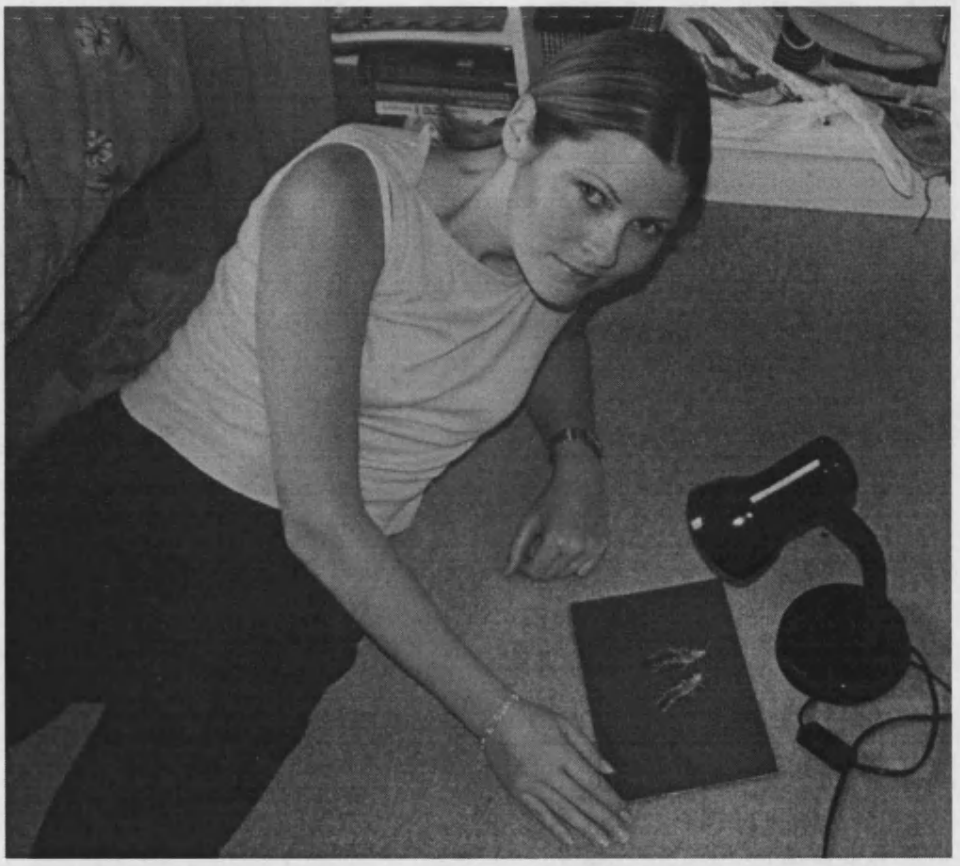
K = [970.8 x 12/15 (16)] – 3 (16)

= 1.01

1.01 to 2 d.f (degrees of freedom) is less than the p = 0.001 tabulated value of 9.21, so we therefore accept the null hypothesis that there is no significant difference between the averages.

xi Appendix III

Original amino acid analysis results



THE UNIVERSITY OF BIRMINGHAM

Alta Bioscience

[s3955 S Jarrold]

Amino Acid Analysis

22 June, 1999

Concentration n.mole/sample free amino acids in solution

Alta code	A3955				
Sample name	**Sample A**				
Asp	3.1				
Thr	11				
Ser	12				
Glu	21				
Pro	18				
Gly	73				
Ala	29				
Cys	0.1				
Val	7.6				
Met					
Ile	4.3				
Leu	4.6				
Tyr	5.5				
Phe	1.7				
His	7.5 *				
Lys	5.1				
Arg	4.1				

* other material eluting close by

Notes about amino acid analysis:- Asn and Gln are completely converted to Asp and Glu during the acid hydrolysis of the protein. The values for Thr and Ser have been corrected for hydrolysis losses of 5% and 10% respectively. Trp usually suffers complete loss during acid hydrolysis and is not normally quantified. In proteins, Cys is usually observed as cystine. The recovery of Cys is variable when using standard hydrolysis conditions. Values are reported to two significant figures.

Approved by _____ Date 22/6/99

Director, Alta Bioscience: Dr J. E. Fox Tel: 0121 414 5450 Fax: 0121 414 3376 Email: altabio@bham.ac.uk

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Alta Bioscience

[a3957 S Jarrold]

Amino Acid Analysis

28 June, 1999

Concentration n.mole/sample free amino acids

Alta code	A3957				
Sample name	**B**				
Asp	0.3				
Thr					
Ser	0.5				
Glu	0.4				
Pro	0.2				
Gly	0.9				
Ala	2.0				
Cys					
Val	0.7				
Met					
Ile	0.4				
Leu	0.8				
Tyr					
Phe	0.3				
His					
Lys	0.1				
Arg					

These values are close to background levels

Notes about amino acid analysis:- Values are reported to two significant figures.

Approved by _____ Date 28/6/99

Director, Alta Bioscience: Dr J. E. Fox Tel: 0121 414 5450 Fax: 0121 414 3376 Email: akabiox@bham.ac.uk

THE UNIVERSITY OF BIRMINGHAM

Alta Bioscience

[s3956 S Jarrold]

Amino Acid Analysis

22 June, 1999

Concentration n.mole/sample after hydrolysis, 24 hours at 110°C

Alta code	A3956				
Sample name	**Sample A**				
Asp	14				
Thr	9.0				
Ser	15				
Glu	51				
Pro	41				
Gly	110				
Ala	95				
Cys					
Val	22				
Met	2.3				
Ile	12				
Leu	19				
Tyr	12				
Phe	3.1				
His	11 *				
Lys	7.1				
Arg	6.6				

* other material eluting close by

Notes about amino acid analysis:- Asn and Gln are completely converted to Asp and Glu during the acid hydrolysis of the protein. The values for Thr and Ser have been corrected for hydrolysis losses of 5% and 10% respectively. Trp usually suffers complete loss during acid hydrolysis and is not normally quantified. In proteins, Cys is usually observed as cystine. The recovery of Cys is variable when using standard hydrolysis conditions. Values are reported to two significant figures.

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THE UNIVERSITY OF BIRMINGHAM

Alta Bioscience

[a3958 S Jarrold]

Amino Acid Analysis

28 June, 1999

Concentration n.mole/sample after hydrolysis, 24 hours at 110°C

Alta code	A3958				
Sample name	**Sample B**				
Asp	0.9				
Thr	0.7				
Ser	1.0				
Glu	1.4				
Pro	1.5				
Gly	4.6				
Ala	3.6				
Cys					
Val	1.3				
Met					
Ile	0.8				
Leu	1.5				
Tyr	0.5				
Phe	0.7				
His	2.4				
Lys					
Arg	0.2				

Notes about amino acid analysis.- Asn and Gln are completely converted to Asp and Glu during the acid hydrolysis of the protein. The values for Thr and Ser have been corrected for hydrolysis losses of 5% and 10% respectively. Trp usually suffers complete loss during acid hydrolysis and is not normally quantified. In proteins, Cys is usually observed as cystine. The recovery of Cys is variable when using standard hydrolysis conditions Values are reported to two significant figures.

Approved by _____ Date 28/6/99

Director Alta Bioscience: Dr J. E. Fox Tel: 0121 414 5450 Fax: 0121 414 3376 Email: altabios@bham.ac.uk